

Chemical Modification and Pharmacological Evaluation of the Antimalarial Natural Product Totarol

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ABSTRACT

Malaria is one of the most predominant infectious diseases in the world. The control of malaria is based primarily on chemoprophylaxis. Over the past couple of decades, resistance to almost every antimalarial has been noted. There is therefore an urgent need to develop antimalarial agents with novel structures and mechanisms of action. Natural products offer a wide variety of biologically validated compounds. These privileged structures hold great potential as scaffolds on which to develop compound libraries.

This dissertation aims to develop promising antiplasmodial lead compounds through chemical modification and structure-activity relationship studies of a range of derivatives, based on the antiplasmodial natural product totarol.

A number of β -amino alcohol derivatives of totarol were synthesized. Two of these derivatives showed potential as possible leads. In order to determine the role of the totarol scaffold in the observed *in vitro* antiplasmodial activity of the lead compounds, analogues, in which the totarol backbone was replaced with simpler aromatic groups, were synthesized.

All derivatives and analogues were characterized using analytical and spectroscopic techniques and evaluated *in vitro* for antiplasmodial activity against a chloroquine sensitive (D10) strain of *Plasmodium falciparum*. Only those showing promising activities ($IC_{50} < 14 \mu M$) were tested against a resistant (K1) strain. The cytotoxicity of promising compounds was determined using a mammalian cell line. Active compounds, with potential stomatogenic features, were investigated at sublytic concentrations to determine their effect on erythrocytic shape. The results, in conjunction with the cytotoxicity results, indicate selective antiplasmodial activity.

Owing to the structural similarities, characteristic to those found in resistance modulators, displayed by the lead compounds, their effect on the uptake and potentiation of chloroquine was examined. One of the compounds was found to increase chloroquine

accumulation, although both displayed antagonistic behavior when combined with chloroquine.

An investigation, prompted by recent literature, into the ability of selected derivatives and analogues to inhibit erythrocytic invasion by merozoites was performed. One pair of analogues was found to significantly inhibit reinfection of erythrocytes.

Analysis of structure-activity relationships suggests chemical features and moieties favorable for selective antiplasmodial activity. The study resulted in four potential lead compounds being synthesized, all possessing novel structures. Two of the leads demonstrated a novel mechanism of action in a preliminary investigation.

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ABBREVIATIONS

APAD	3-acetylpyridine adenine dinucleotide
APCI	atomic pressure compound ionization
CDCl_3	deuterated chloroform
CHCl_3	chloroform
CHO	Chinese hamster ovarian cells
COSY	correlation spectroscopy
CQ	chloroquine
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthetase
DMEM	Dulbecco's Modified Eagles' Medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
d-TMP	deoxythymidine monophosphate
EDTA	tetrasodium salt of ethylenediaminetetraacetic acid
EIMS	electron impact mass spectrometry
Et_2O	diethyl ether
EtOAc	ethyl acetate
Fe(III)PPIX	ferriprotoporphyrin IX
FCS	fetal calf serum
g	gram(s)
Hex	hexane
HMBC	heteronuclear multiple bond correlation
HPLC	high pressure liquid chromatography
HSQC	heteronuclear spin correlation through multiple quantum coherence
Hz	hertz
IC_{50}	50% inhibitory concentration
IR	infrared spectroscopy
J	coupling constant
LREIMS	low resolution mass spectrometry
M	molar

MDR	multidrug resistant
MeOH	methanol
mg	milligram
MgSO ₄	magnesium sulphate
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide
NaH	Sodium Hydride
ND	Not determined
NMR	nuclear magnetic resonance
PABA	<i>p</i> -aminobenzoic acid
<i>Pfcr</i>	<i>P. falciparum</i> chloroquine resistance transporter
<i>pfmdr</i>	<i>P. falciparum</i> homologues of the human <i>mdr</i> -type gene
ppm	parts per million
pRBC	parasitised erythrocyte
RBC	erythrocyte
R _i	resistance index
RMI	response modification index
rpm	revolutions per minute
S _i	selectivity index
THF	tetrahydrofuran
TLC	thin layer chromatography
μg	microgram(s)
μM	micromolar

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Chapter 1

Malaria and Project Background

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1.1. Introduction

Malaria is one of the most predominant infectious diseases in the world. There are up to 500 million acute cases of malaria and a mortality rate of over a million people reported annually [Greenwood, 2004]. Malaria is an ancient disease, and was found to be reported as early as 2700 B. C. [Oaks *et al.*, 1991]. The name malaria, was only used after the mid-eighteenth century, and is derived from the Latin, *mal aria*, meaning “bad air” [Dutta and Dutt, 1978]. It was established that there was a link between the “bad air” and stagnant water, but Hippocrates was first to identify a connection between the stagnant water and malaria-like fevers [Oaks *et al.*, 1991]. In 1883, King proposed mosquitoes as the vector for the transmission of malaria. Over a decade later, Ross established that the *Anopheles* mosquito was responsible for the majority of malaria transmission, earning him the Nobel Prize in 1902 [Dutta and Dutt, 1978].

Malaria is a protozoan parasitic disease caused by four species belonging to the *Plasmodium* genus, namely *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* [Frédérich *et al.* 2002].

The onset of disease occurs 8-30 days after infection and is characterized by a flu-like illness with cycles of fever. Headaches, muscle aches, weakness, vomiting, diarrhea and coughing may also be present. The time period between the fever cycles is dependent on the species of parasite. The most severe form of malaria is associated with *P. falciparum*, which can cause cerebral malaria resulting in death if untreated [www.who.int/inf-fs/en/InformationSheet01.pdf].

1.2. Life cycle of the malaria parasite

The hematophagous anthropophilous female *Anopheles* mosquito is the vector through which the malaria parasite is transmitted to humans. Out of approximately 300 species of the *Anopheles* genus, 85 are malaria vectors. Only the female *Anopheles* is hematophagous, and possesses mandibles and maxillae for this purpose, as it is necessary for the female to have a blood meal before reproduction [Dutta and Dutt, 1978]. Humans are the intermediate hosts, in which the asexual reproductive stage of the parasite life cycle takes place [Moore *et al.*, 2002]. The sexual reproductive stage occurs within the midgut of the mosquito [Frédérich *et al.* 2002] (Figure 1.1.1.).

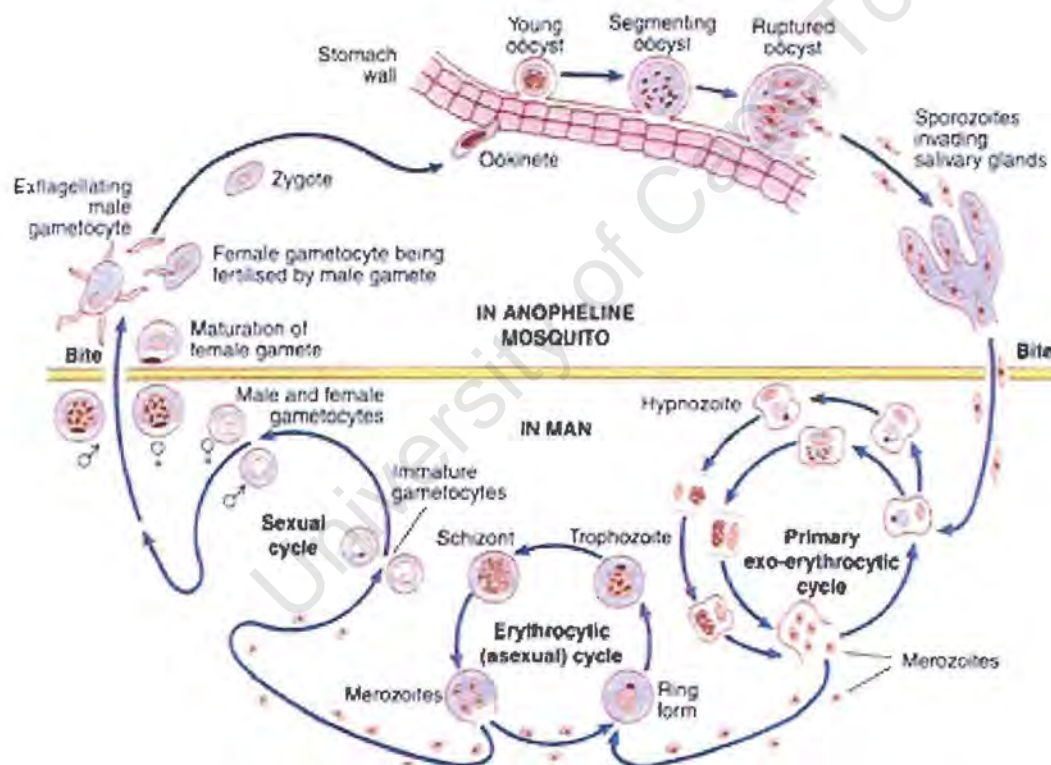


Figure 1.1.1.: Life cycle of the malaria parasite [www.malariasite.com/malaria/LifeCycle.htm].

1.2.1. Sexual (Sporogonic) phase

This phase of the life cycle occurs within the *Anopheles* mosquito. It is initiated when the mosquito feeds on a human host with erythrocytic stage malaria. Gametocytes are ingested and develop into male microgametes and female macrogametocytes within the mosquito's gut. Fertilization occurs, after which the zygote encysts in the gut wall and forms an oöcyst. This oöcyst produces sporozoites, which are released upon rupture and migrate to the salivary glands for transmission to a vertebrate host (in this case a human) during the mosquito's next blood meal [Dutta and Dutt, 1978].

1.2.2. Asexual (Schizogonic) phase

This stage can be further divided into an exoerythrocytic or hepatic phase and an erythrocytic phase [Frédérich *et al.* 2002]. The exoerythrocytic phase is initiated with the transmission of sporozoites from an infected mosquito into a human. The sporozoites migrate to the liver where they develop into schizonts. The rate of schizont formation and development is dependent on the *Plasmodium* species. *P. vivax* and *ovale* form hypnozoites, which may remain dormant in the liver for several years, before developing into schizonts. *P. falciparum* and *malariae* do not form hypnozoites and development of schizonts takes approximately 1 to 2 weeks [Frédérich *et al.*, 2002]. Merozoites are released upon schizont rupture and invade the erythrocytes, initiating the erythrocytic phase of the parasite's life cycle. Parasite invasion of the erythrocyte is complex, and involves parasitic and erythrocytic receptor interactions [Chitnis and Blackman, 2000]. Within the erythrocytes the merozoites undergo asexual reproduction, forming rings and trophozoites, and finally erythrocytic schizonts. Lysis of the erythrocyte at this stage releases merozoites that either invade new red blood cells or differentiate into gametocytes. Continuation of the erythrocytic phase is maximized by collective rupturing of infected erythrocytes and the short time period (30 seconds) of erythrocytic invasion, which overwhelms the immune system [Frédérich *et al.*, 2002]. Gametocytes are transmitted to a female *Anopheles* mosquito during feeding, hence completing the cycle [Frédérich *et al.*, 2002]. The whole erythrocytic cycle takes 48 hours for tropical (*P. falciparum*) and tertian (*P. vivax*) malaria and 72 hours for quartan malaria (*P. malariae*). The onset of illness and

fever occur upon rupture of infected erythrocytes, due to the release of plasmodial waste products and other foreign substances into the blood stream [Dutta and Dutt, 1978].

1.3. Distribution

Malaria is found in tropical and subtropical areas throughout the world, where the temperature is above 16°C - the minimum temperature at which development of the malaria parasite occurs [Sachs and Malaney, 2002] (Figure 1.3.1.). The most widely affected area is Sub-Saharan Africa.

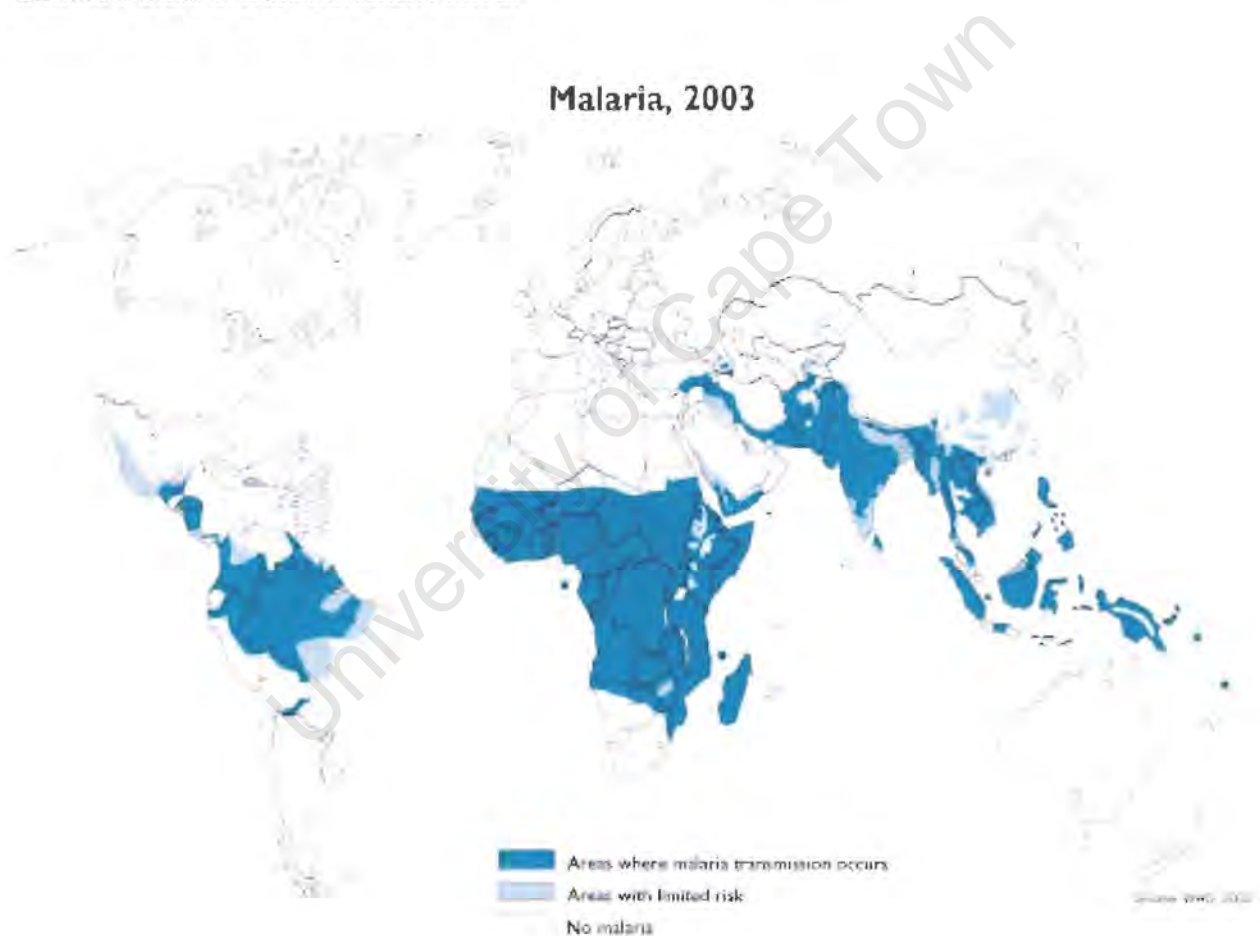


Figure 1.3.1.: Global distribution of malaria [www.who.int/ith/chapter05_m08_malaria.html].

Malaria in South Africa is found mainly in the northern regions of low altitude, such as Mpumalanga, Northern Province and northeastern KwaZulu-Natal [www.malaria.org.za/body_prophylaxiscontents/Intro/intro.htm] (Figure 1.3.2.).

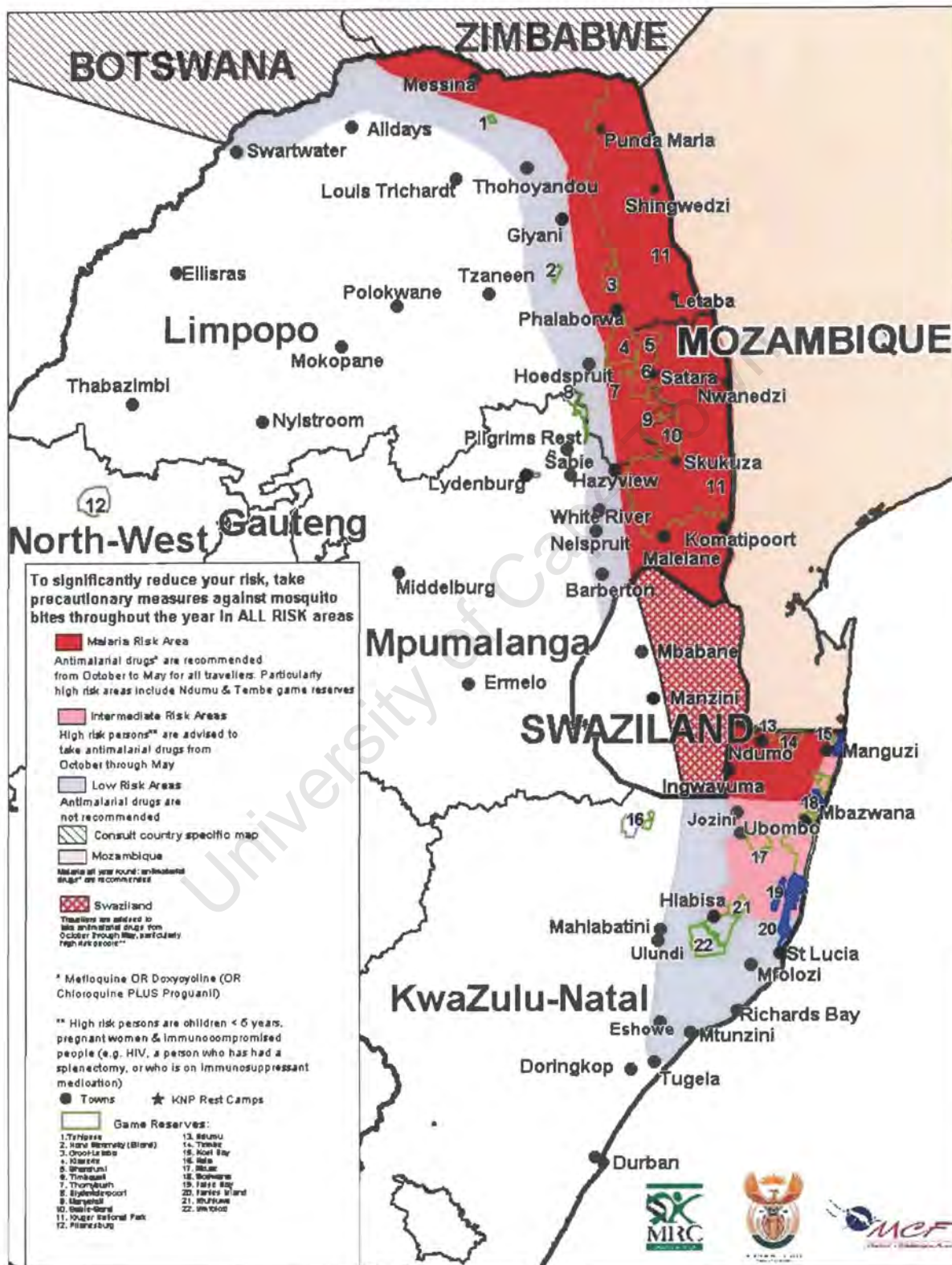


Figure 1.3.2.: Regions in South Africa where malaria occurs [www.mic.uct.ac.za/map.php].

As already mentioned, Sub-saharan Africa is the largest area to be affected with malaria and has the vast majority of malaria-related deaths. The reason for this is due to several reasons such as climate [Dutta and Dutt, 1978; www.who.int/inf-fs/en/1InformationSheet03.pdf], vector species [Winstanley, 2001], flora and fauna, land-use, resources and infrastructure as well as rapidly developing resistance to first-line drugs and insecticides. The impact and factors influencing the spread of malaria, with special reference to Africa, will be discussed in the following section.

1.4. The impact of malaria

The devastating effects of malaria are especially noticeable in countries or regions with high infection rates, such as sub-Saharan Africa. It is estimated that malaria is responsible for 2000 deaths daily (mostly children) worldwide, 90% of these being in Africa. The disease places a huge burden on the underdeveloped health care systems and available resources in these countries, and many eradication campaigns have failed due to lack of funding and infrastructure [www.who.int/inf-fs/en/1InformationSheet03.pdf].

Malaria has a great impact on social and economic development, as seen in the global distribution of per-capita gross domestic product (GDP), which is lowest in malaria-infected countries. The economic growth is also substantially less in these areas. The explanations for the correlation between malaria and poverty are numerous. Malaria influences development by affecting fertility, mortality, population growth, productivity, absenteeism, underdevelopment of children, fetal development, investments and medical costs. Lack of both personal and government funds to spend on malaria prevention and cure aids transmission and mortality [Sachs and Malaney, 2002].

1.5. Factors influencing the spread of malaria

There are many factors that affect the spread of malaria. These can be divided up into two broad groups, namely; i) social and environmental factors and ii) resistance of both the mosquitoes to insecticides and the parasites to chemoprophylactics.

1.5.1. Social and environmental factors

Social factors such as war, population growth, tourism and HIV infection [Rowland-Jones and Lohman, 2002] have led to a resurgence of the disease, by increasing the number of susceptible individuals and communities [Greenwood and Mutabingwa, 2002] and by decreasing very limited funding that would previously have been used for malaria prevention and control.

Environmental changes and ecological disturbances also have a profound effect on the resurgence of malaria. Both these phenomena shift the ecological balance and therefore affect the population growth of host, vector and parasite. Examples of disturbances caused by man are deforestation, land use changes and building of dams and other water control projects. Human movement also plays a role as it increases the number of migrants who would not normally be exposed to malaria and therefore have no innate immunity or knowledge of ways to minimize infection. Environmental factors, such as climate, temperature, rainfall and El Niño also influence the spread of the disease, by making areas, which are non-endemic, suitable for vector growth and thus parasite transmission [Patz *et al.*, 2000].

1.5.2. Resistance

The failure of vector control programs and developing resistance of mosquitoes to insecticides places increasing reliance on antimalarial chemotherapy. Today chemotherapy is the biggest weapon in the fight against malaria, although resistance to virtually all antimalarials has been noted [Campbell, 1997]. Resistance is defined as the “ability of a parasite strain to survive and/or multiply despite the administration and

absorption of a drug given in doses equal to, or higher than, those usually recommended but within the limits of tolerance of the subject" [WHO, 1973]. Chloroquine-resistant *falciparum* malaria was first noted in Southeast Asia and South America in the early 1960's. By the late 1980's resistance to sulfadoxine-pyrimethamine and to mefloquine was also reported [Oaks *et al.*, 1991; Wongsrichanalai *et al.*, 2002]. There are effective drugs available, such as artemisinin and its derivatives, but these are very expensive and are therefore not viable options for developing countries [Wongsrichanalai *et al.*, 2002].

Many factors contribute to the development and spread of resistance. Selection and spread of resistant parasites occurs under drug pressure and non-compliance, such as under-dosage or treatment not being completed. Characteristics of the drug are also important factors in the development of resistance. Drugs with long half-lives, such as mefloquine, may select for resistant strains as they will be present in the body at sub-therapeutic levels after treatment and/or cure of an infection. Recrudescence at this stage will select for resistant parasites. Widespread, intense usage of drugs can also increase drug selection pressure, thereby favoring resistant strain development [Wongsrichanalai *et al.*, 2002].

1.6. Preventative measures

1.6.1. Vector control

Vector control after World War II consisted of draining mosquito breeding grounds (i.e. stagnant water such as swamps), screens and spraying with DDT. In 1955, the Eighth World Health Assembly launched a plan, the objective of which was worldwide eradication of malaria. This plan was successful in many countries such as Greece, Venezuela and the USA. Unfortunately malaria eradication was not achieved in Africa, due to poor project planning, infrastructure and developing resistance to insecticides [McMillan and Meltzer, 1996]. Owing to these failures, the plan for eradication of malaria through vector control was abandoned in the 1960s [Guerin *et al.*, 2002]. This meant withdrawal of international funding, and resulted in devastating consequences for developing countries, which do not have the funding and resources available to launch such campaigns. The use of DDT after

this period was strictly controlled owing to its impact on the environment [Oaks *et al.*, 1991] and fear of further resistance development. Insecticide-treated nets now play a vital role in vector control, although their effectiveness is debatable [McMillan and Meltzer, 1996]. Malaria control is at present mainly reliant on chemoprophylaxis, due to failure of vector control strategies and other eradication campaigns.

1.6.2. Drug Therapy

Antimalarial drugs can be divided into two types, blood schizontocides, which target the erythrocytic stage of the parasite and tissue schizontocides, which target the liver stage of the parasite [Kumar *et al.*, 2003]. Unlike other forms of malaria, *falciparum* malaria does not form hypnozoites, which can remain dormant and cause reinfection at a later stage. A single blood schizontocide is therefore sufficient as treatment for *falciparum* malaria [Kumar *et al.*, 2003] and these drugs will therefore be focused on.

There are three main groups of commonly prescribed antimalarials which are listed below;

- Antifolates
- Quinolines
- Artemisinin and derivatives

1.6.2.1. Antifolates

Antifolate antimalarials act by inhibiting the dihydrofolate pathway of the parasite. This inhibits folate synthesis, which results in decreased pyrimidine synthesis and ultimately inhibition of DNA synthesis and decreased methionine and serine synthesis, leading to parasite death. There are 2 classes of antifolate antimalarials, inhibitors of dihydrofolate reductase (DHFR) and inhibitors of dihydropteroate synthetase (DHPS) [Casteel, 1997; Foote and Cowman, 1994].

DHFR inhibitors

DHFR exists as a bifunctional enzyme in protozoa and reduces the dihydrofolate (DHF) produced in d-TMP (deoxythymidine monophosphate) synthesis and *de novo* folate

synthesis and salvage pathways. DHFR inhibitors, pyrimethamine and cycloguanil selectively inhibit *P. falciparum* DHFR (due to structural and functional differences between *P. falciparum* and human DHFR), thereby disabling the reduction of DHF and therefore tetrahydrofuran (THF) formation, an essential cofactor in a number of vital reactions, such as synthesis of certain amino acids and purine nucleotides [Olliaro, 2001; Yuthavong, 2002]. DHFR inhibitors mimic the pteridine ring of DHF and compete with it for the active site of DHFR. The compound fits into the hydrophobic active site and is stabilized via hydrogen bonding with Asp54 and hydrophobic interactions with residues 108, 16, 51, 59 and 164. DHF is a flexible molecule due to its bridging alkyl chain (Figure 1.6.2.1.1.), which allows the amino benzyl glutamate portion of the molecule to be perpendicular to the pteridine ring, when it is bound by DHFR. This is unlike DHFR inhibitors, which tend to be rigid and it has been proposed that this rigidity may play a role in resistance [Olliaro, 2001; Warhurst 1998].

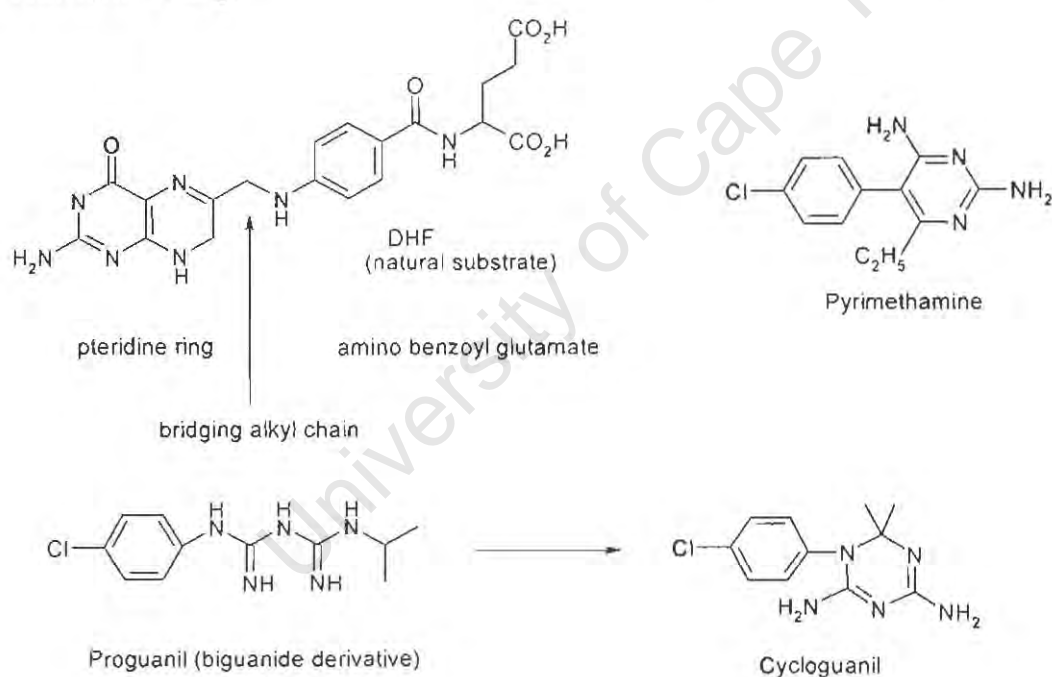


Figure 1.6.2.1.1.: Structure of DHF and DHFR inhibitors pyrimethamine, cycloguanil, proguanil [Casteel, 1997; Frédérich *et al.*, 2002].

Proguanil is believed to be a prodrug of cycloguanil, and is converted to its active form by cytochrome P450. The efficacy of cycloguanil/proguanil is therefore affected by genetic polymorphisms in the P450 enzymes [Casteel, 1997]. A recent paper by Sweeney *et al.*

(2003), suggests that the antimalarial action of proguanil is not solely responsible for the antifolate action of the cycloguanil. They suggest that proguanil, a biguanide derivative, binds to endogenous metals forming a complex with inhibitory falcipain-2 activity. Falcipain-2 is a cysteine protease involved in haemoglobin degradation [Shenai *et al.*, 2000], which is necessary for parasitic protein synthesis [Banerjee and Goldberg, 2001]. Cysteine protease inhibitors completely prevent haemoglobin degradation, preventing protein synthesis and hence parasite growth [Rosenthal *et al.*, 1993; Shenai and Rosenthal, 2002].

Structure Activity Relationships

Molecular modeling is being used to mimic the active site of DHFR and allows predictions of essential characteristics, as seen in Figure 1.6.2.1.2., needed in inhibitors for optimum binding and hence efficacy [Warhurst, 1998].

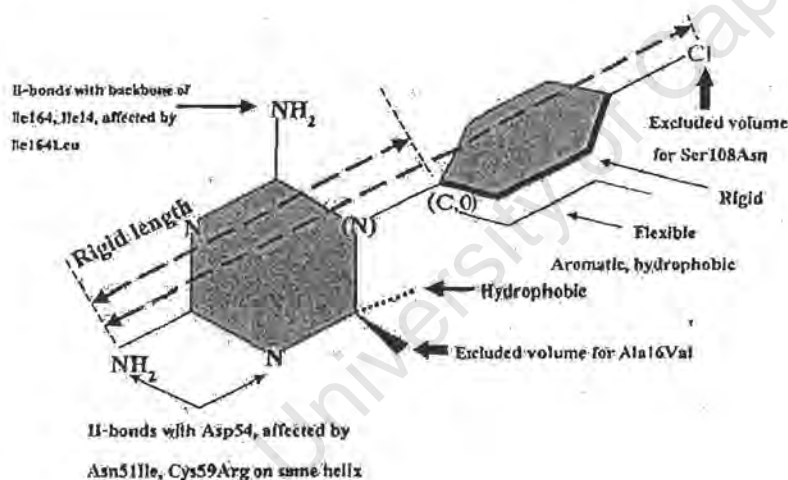


Figure 1.6.2.1.2.: Essential features of a *P. falciparum* DHFR inhibitor [Yuthawong, 2002].

DHPS inhibitors

DHPS is also a bifunctional enzyme in protozoa and combines *p*-amino benzoic acid (PABA) with a pterin derivative to form dihydropteroate. Inhibitors are highly selective for *P. falciparum* DHPS and inhibit DHF formation by mimicking PABA (Figure 1.6.2.1.3.) [Warhurst, 1998].

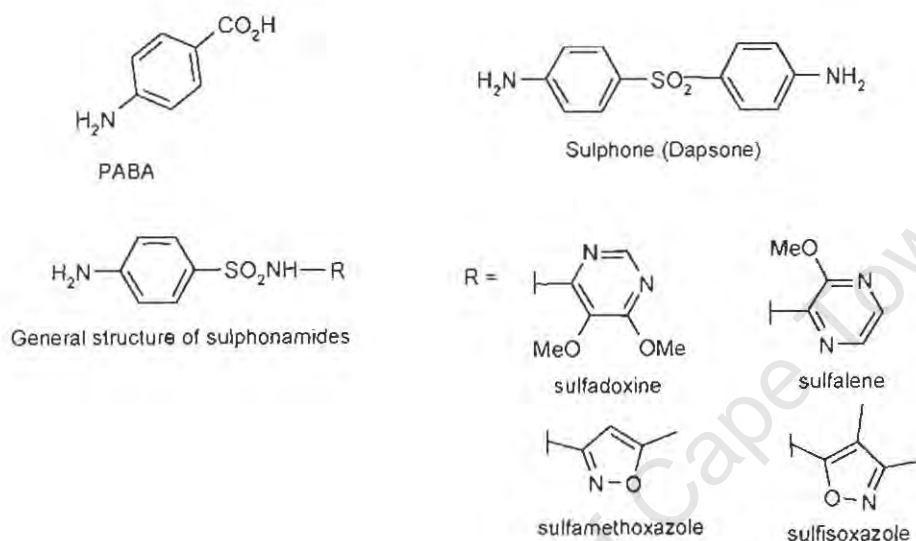


Figure 1.6.2.1.3.: Structure of *p*-aminobenzoic acid (PABA) [Clayden *et al.*, 2001], a sulphone [Bruce-Chwatt *et al.*, 1981] and various sulphonamides [Casteel, 1997].

Resistance to antifolates

Resistance is widespread and limits the use of this drug class. Resistance can be attributed to point mutations in the DHFR and DHPS genes as shown in Figure 1.6.2.1.4. [Biagini *et al.*, 2003; Olliaro, 2001; Yuthavong, 2002].

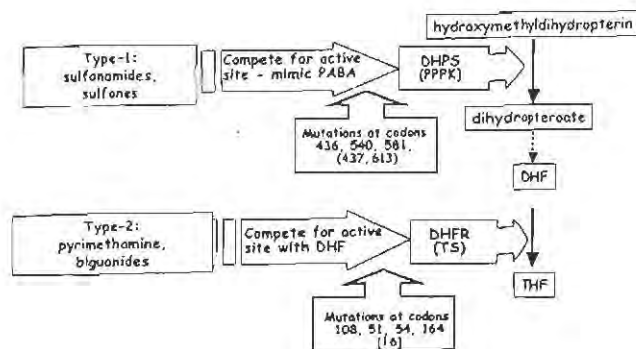


Figure 1.6.2.1.4.: Targets of and mutations conferring resistance to DHFR and DHPS inhibitors [Olliaro, 2001].

1.6.2.2. Quinoline-containing antimalarials

Quinoline-containing antimalarials (Figure 1.6.2.2.1.) can be split into 2 subclasses:

- 4-aminoquinolines
- Aryl-amino alcohols

Both subclasses act on the parasite's endolysosomal system, differing in their interaction with their putative targets [Warhurst, 1987] and have inversely related parasite sensitivities [Fitch, 2004; Olliaro, 2001]. The 4-aminoquinoline subclass includes chloroquine (CQ), amodiaquine and pyronaridine. These compounds are all weak bases and are diprotonated and hydrophilic at neutral pH. The aryl-amino alcohol subclass consists of quinine, quinidine, mefloquine and halofantrine. These compounds are weaker bases than the 4-aminoquinolines and are hydrophobic (lipid soluble) at neutral pH [Olliaro, 2001].

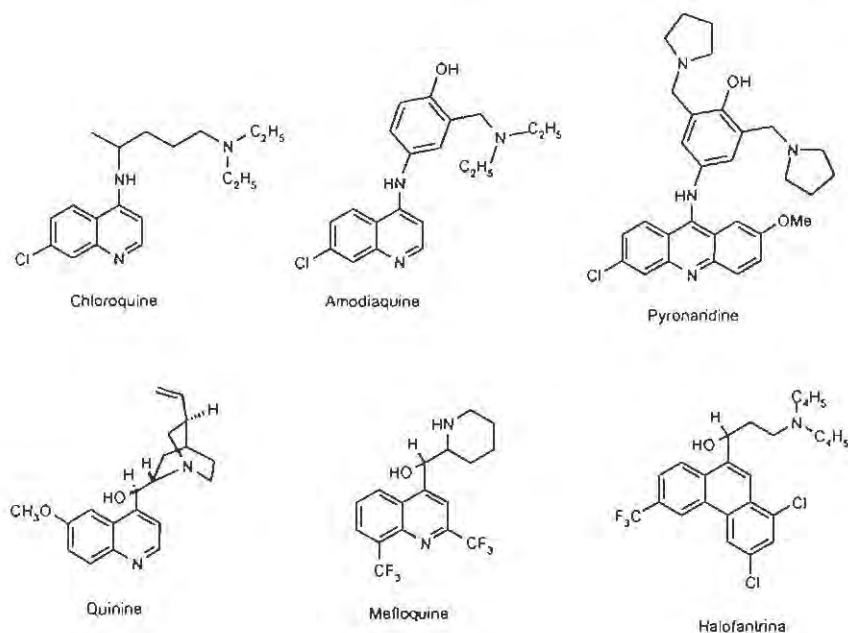


Figure 1.6.2.2.1.: Structures of various 4-aminoquinoline and aryl-amino alcohol antimalarials.

Background

Quinine, a component of the *Cinchona* bark, was the first antimalarial to be discovered and was used by South American Indians as a treatment for malaria fevers [Oaks *et al.*, 1991]. Chemical modification of quinine, in order to develop less toxic derivatives, led to the synthesis of the 4-aminoquinoline, chloroquine [Loeb *et al.*, 1946]. Investigation into the structure-activity relationship of various 4-aminoquinolines lead to the synthesis of amodiaquine [O'Neill *et al.*, 1998]. Development of resistance to the 4-aminoquinolines prompted the development of the aryl-amino alcohols, mefloquine and halofantrine [Casteel, 1997; Foley and Tilley, 1997].

Mechanism of action

Quinine-containing antimalarials act on the mature (i.e. trophozoite) stages of the asexual blood phase of the parasite's life cycle. In this phase, parasites degrade haemoglobin, obtained from the host erythrocyte's cytoplasm, to amino acids which are subsequently

used to synthesize proteins. This is necessary as intraerythrocytic stages of *P. falciparum* are able to synthesize very few amino acids *de novo* [Sherman, 1977]. Haemoglobin degradation occurs in excess of the amount needed for sufficient amino acid production. This excess degradation serves to reduce the colloid-osmotic pressure within the host cell, thereby maintaining the osmotic stability of the infected erythrocyte [Lew *et al.*, 2004]. Haemoglobin degradation is thought to be an ordered event involving a number of classes of enzymes. Plasmeepsins I, II and IV (aspartic proteases and histo-aspartic protease) [Banerjee *et al.*, 2002] initiate haemoglobin hydrolysis, and produce denatured haemoglobin which is then cleaved into small peptides by falcipains -2 and -3 (cysteine proteases) [Shenai *et al.*, 2000; Sijwali *et al.*, 2001] and falcilysin (metalloprotease) [Banerjee and Goldberg, 2001; Gluzman *et al.*, 1994; Kolakovich Eggleson *et al.*, 1999]. These small peptides are then transported to the parasite cytoplasm where they are hydrolyzed into amino acids [Kolakovich *et al.*, 1997]. Ferriprotoporphyrin IX (FP) is a toxic byproduct of haemoglobin degradation, and is sequestered by the parasite in crystalline non-toxic structures called haemozoin [Cowman, 1995; Fitch, 2004; Foote and Cowman, 1994].

There have been a number of theories regarding the mechanism of action of the 4-aminoquinoline antimalarials, such as DNA interaction [Cohen and Yielding, 1965; O'Brien *et al.*, 1966], inhibition of protein synthesis [Sirolia and Padmanaban, 1991], inhibition of vacuolar phospholipase [Ginsburg and Geary, 1987] and lysosomotropism [De Duve *et al.*, 1974]. However, the most widely accepted hypothesis proposes that 4-aminoquinolines exert their specific antimalarial activity by interfering with haemozoin formation [Fitch, 1986]. The quinoline methanols have been shown to have a related mechanism of action to the 4-aminoquinolines although the precise mechanism by which this occurs is still uncertain [Egan, 2004].

Structure Activity Relationships

4-Aminoquinolines

The two most common examples of this subclass, CQ and amodiaquine, will be discussed.

General: The aminoquinoline ring is responsible for complexing ferriprotoporphyrin IX (Fe(III)PPIX). It was shown that strong complexes of the compound and Fe(III)PPIX are only formed by the 2- and 4-aminoquinolines, possibly because only 2- and 4-aminoquinolines possess unique resonance forms [Egan *et al.*, 2000]. The 4-arylamino moiety however, enhances the activity of derivatives against CQ resistant strains. Comparison of the pKa values of CQ and amodiaquine with other aminoquinolines, reveals that both these compounds possess relatively high pKa values. It has been suggested that the formation of Fe(III)PPIX-chloroquine complex involves cation- π interactions, although complex formation is pH insensitive [Kaschula *et al.*, 2002].

The removal of the Cl group at the 7-position of the quinoline ring abolishes the antiparasmodial activity of the respective compounds. It is believed that the 7-chloro group is required for the inhibition of β -haematin formation [Egan *et al.*, 2000]. Experiments were performed in which the 7-chloro group was replaced with various other substituents. It was determined that lipophilicity, hydrophilicity or electronegativity at position 7 are not significant factors in the antimalarial activity [De *et al.*, 1998]. Other modifications on the quinoline ring, of 4-aminoquinolines, such as the oxidation of the nitrogen atom, results in compounds that display a high level of antiparasmodial activity [Elslager *et al.*, 1964].

The basic amino side chain is thought to be necessary for accumulation of the drug in the food vacuole of the parasite [Egan *et al.*, 2000] and to assist binding to haem. Accumulation is thought to occur firstly via the weak base effect and then via pH trapping [O'Neill *et al.*, 1997].

The number of carbons between the two nitrogens in the diaminoalkane side chain also influences the activity of these compounds. It was found that side chains with 2-12 carbons are comparable with the activity of chloroquine on chloroquine-sensitive strains of *P.*

falciparum. Aminoquinolines with short (2-3 carbon) or long (10-12 carbon) diaminoalkane side chains however, were found to be active against CQ sensitive, resistant and multiply resistant *P. falciparum* *in vivo* [De *et al.*, 1996; Ridley *et al.*, 1996]. Intermediate (tetra-, penta-, hexa- and octa-methylene) diaminoalkane side chains on the other hand, were active against CQ sensitive and not CQ resistant *P. falciparum* [De *et al.*, 1998]. Bray *et al.* (1996), observed that dealkylation increases cross resistance and verapamil resistance reversal of the major metabolites of both CQ and amodiaquine. They also proposed that compounds with a log D (lipid solubility) of 1.8-3 at a pH of 7.2 with a tertiary amino moiety 0.6-0.9 nm from the ring nitrogen would have activity in the nanomolar range and display minimal cross resistance with CQ.

CQ: CQ contains a 7-chloroquinoline substituted ring and a diamine side chain [O'Neill *et al.*, 1998]. It has been observed that short, 2 carbon side chain CQ analogues have *in vitro* activity (<15nM) against CQ resistant strains but undergo *N*-terminal dealkylation *in vivo*, producing less active metabolites [Biagini *et al.*, 2003].

Figure 1.6.2.2.2. summarizes the structure-function relationship of chloroquine, although similar features confer the same properties in amodiaquine.

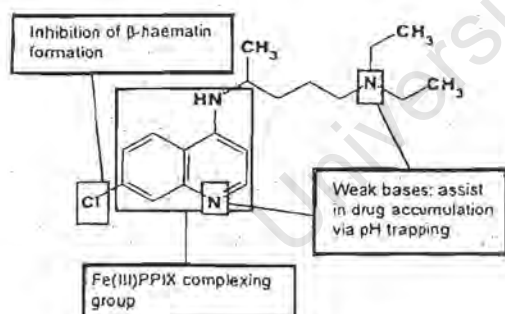


Figure 1.6.2.2.2.: Structure-function relationship in CQ [Egan *et al.*, 2000].

Amodiaquine: Amodiaquine is hepatotoxic and causes agranulocytosis. This is not surprising, as the amodiaquine side chain contains a 4-aminophenol, which undergoes metabolic oxidation to produce quinoneimines [O'Neill *et al.*, 1998]. This aromatic hydroxyl group does however increase antiparasmodial activity [Biagini *et al.*, 2003].

Aryl-amino alcohols

These compounds accumulate in the acidic food vacuole of the parasite due to pH trapping [Yayon *et al.*, 1984]. Here the compounds are thought to complex to haem, and in so doing are toxic to the parasite. This complexation to haem is reliant on the quinoline ring. It has also been noted that amino alcohol antimalarials require an aromatic and amino moiety separated by 2-3 carbon atoms for activity [Bhattacharjee and Karle, 1996].

Resistance

CQ resistance was first noted in Southeast Asia and South America in the late 1950's, and led to many theories on the mechanisms of resistance [Batra and Bhaduri, 1997; Olliaro, 2001; Thaitong *et al.*, 1983; Warhurst, 1988]. One of the first developments into the mechanism of resistance to CQ was the finding that resistant parasites accumulate significantly less CQ than sensitive ones [Fitch 1970; Verdier *et al.*, 1985]. This led to the assumption that resistance could be mediated through either increased efflux, decreased influx or a combination of both [Foote and Cowman, 1994; O'Neill *et al.*, 1998]. Further research has shown this decrease in CQ concentration to be a factor in resistance, although it is generally accepted that multiple gene mutations and mechanisms confer resistance.

Decreased prophylactic concentration, cross-resistance between multiple structurally unrelated drugs and the observation that verapamil, as well as other structurally and chemically related compounds, could modulate *P. falciparum* resistance to CQ (see section on resistance reversal) [Martin *et al.*, 1987; Zamora *et al.*, 1988], led to the assumption that the cross resistance seen in *P. falciparum* is similar to that seen in multidrug resistant (MDR) cancer cells [Martin *et al.*, 1987]. This led to the hypothesis that the resistance of *P. falciparum* may be due to similar mechanisms to those seen in cancer cells [Bray and Ward, 1998]. P-glycoproteins are membranous proteins thought to be involved in ATP-dependent drug transport and have been reported to mediate MDR [Hayes and Wolf, 1990], via enhancing efflux of chemoprophylactics. Two P-glycoprotein homologues were identified in *P. falciparum* namely, *pfmdr1* and *pfmdr2* (*P. falciparum*

homologues of the human *mdr*-type gene) [Foote *et al.*, 1989; Wilson *et al.*, 1989]. Polymorphisms in *pfmdr1* were linked to the CQ-resistance phenotype [Foote *et al.*, 1990]. The P-glycoprotein homologue, Pgh1, is expressed by *pfmdr1* and is localized in the food vacuole membrane [Cowman *et al.*, 1991] although its expression does not correlate with CQ resistance [Basco *et al.*, 1995; Basco *et al.*, 1996; Wellem's *et al.*, 1990; Wilson *et al.*, 1993]. CQ resistance has also been reported to be attributable to mutations in, or overexpression of the *Pfcr1* (*P. falciparum* chloroquine resistance transporter) gene, which codes for an energy-dependent channel or transporter in the food vacuole membrane [Fidock *et al.*, 2000; Sanchez *et al.*, 2003].

Polymorphisms, amplification and overexpression of *pfmdr1*, seem to correlate with mefloquine, quinine and halofantrine resistance [Wilson *et al.*, 1993; Cowman *et al.*, 1994; Foley and Tilley, 1997], as well as sensitivity to CQ (strain-specific), artemisinin [Reed *et al.*, 2000] and artesunate [Price *et al.*, 2004]. This suggests that PfPgh-1 (*P. falciparum* P-glycoprotein homologue) is involved in conferring resistance to amino alcohol antimalarials [Foley and Tilley, 1997], although resistance to the amino alcohols is not reversed by verapamil, as one would expect if PfPgh-1 was responsible for resistance, but by a neuroleptic [Oduola *et al.*, 1993]. Cross-resistance between the aryl-amino alcohol antimalarials [Cowman *et al.*, 1994] suggests similar mechanisms of resistance to the individual drugs within this class [Gay *et al.*, 1990]. The resistance between aryl-amino alcohol antimalarials and CQ has been shown to have an inverse relationship [Cowman *et al.*, 1994].

Resistance Reversal

Resistance reversal agents or chemosensitizers are agents which increase the sensitivity of a resistant strain to a chemoprophylactic to a similar level observed in the sensitive strain at sub-toxic concentrations. Martin *et al.* (1987), demonstrated that verapamil (Figure 1.6.2.2.3.), a Ca^{2+} antagonist and established resistance reversal agent in neoplastic cells, reversed CQ resistance in *P. falciparum* at sub-inhibitory concentrations. This verapamil induced reversal is not stereospecific, and therefore is unlikely to be mediated through stereospecific receptors or channels [Ye and Van Dyke, 1988].

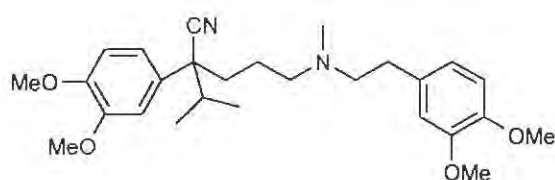


Figure 1.6.2.2.3.: Structure of verapamil

Since this discovery, a number of other compounds have been shown to reverse resistance in *P. falciparum*. In general, all of these compounds have similar structures consisting of a lipid soluble portion, two planar aromatic rings, a cationic charge and a protonatable tertiary nitrogen [Beck, 1990; Rasoanaivo *et al.*, 1996; Zamora *et al.*, 1988].

A number of naturally derived resistance modulator compounds have also been isolated from plants prescribed by traditional healers to enhance CQ activity (CQ-adjuvants). An example of a plant used as a CQ-adjuvant is *Strychnos myrtoides*, from which 2 CQ potentiating compounds, strychnobrasiline and malagashanine were isolated [Rafatro *et al.*, 2000; Rasoanaivo *et al.*, 1994].

1.6.2.3. Artemisinin and its derivatives

Artemisinin or Qinghaosu was isolated from *Artemisia annua*, which was used as a treatment for fevers and chills. Artemisinin is a sesquiterpene trioxane lactone containing a peroxide bridge (Figure 1.6.2.3.1.) [Casteel, 1997; Frédérich *et al.*, 2002]. Due to its low solubility in water and oil, a number of derivatives were synthesized. The reduction of artemisinin yields dihydroartemisinin, from which the first generation analogues (ester/ether derivatives), artesunate, artelinate (water soluble), artemether and arteether (oil soluble), were synthesized [Meshnick *et al.*, 1996]. These compounds have short half-lives and toxicity has been reported [Gordi and Lepist, 2004]. Second-generation derivatives include semi-synthetic and synthetic endoperoxide analogues [Biagini *et al.*, 2003].

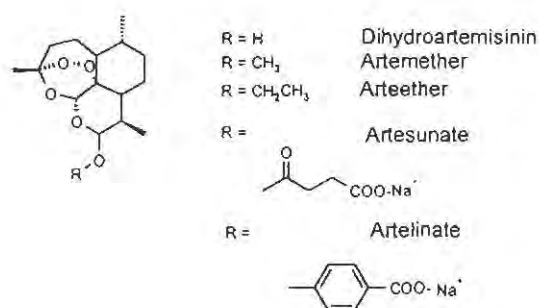


Figure 1.6.2.3.1.: Structure of artemisinin and related first generation analogues.

Mechanism of action

Artemisinin and its derivatives are thought to act by forming carbon-centered radicals in the presence of iron (Fe^{2+}). These radicals selectively target and form covalent adducts with a number of membranous proteins. This mechanism of action as well as the role of the haem iron has recently been disputed [Parapini *et al.*, 2004]. One of the proteins targeted by the artemisinins has been identified as PfATP6, a SERCA (sarco/endoplasmic reticulum Ca^{2+} -ATPase). It is believed that artemisinins inhibit the activity of PfATP6 [Eckstein-Ludwig *et al.*, 2003].

Artemisinin has also been shown to inhibit the activation of the transcriptional factor NF- κ B, thereby preventing induction of nitric oxide synthase transcription and hence nitric oxide synthesis in cytokine-stimulated human astrocytoma T67 cells. High intracerebral nitric oxide levels have been proposed to cause neurological disorders in cerebral malaria. Artemisinin's potent antimalarial activity could therefore be attributed not only to its ability to kill the parasites, but also to its ability to reduce synthesis of nitric oxide [Aldieri *et al.*, 2003].

Structure Activity Relationships

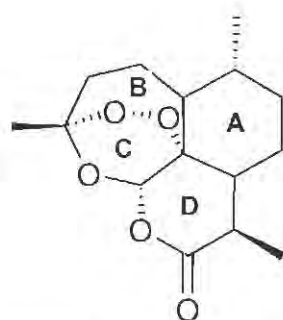


Figure 1.6.2.3.2.: Assignment of artemisinin's rings.

SAR studies performed on artemisinin revealed that its complex ring structure is not necessary for activity. The D ring (Figure 1.6.2.3.2.) is not required, but ring A is necessary for increased activity, probably due to the rigidity it offers the compound. The D ring oxygen, the trioxane ring and the lactol carbon and oxygen also appear important for activity [Casteel, 1997]. The peroxide bridge and available iron were found to be crucial [Casteel, 1997; Eckstein-Ludwig *et al.*, 2003; Meshnick *et al.*, 1996]. The lipophilicity of the artemisinins was also shown to be critical to activity, as an increase in polarity, in order to increase water solubility, decreases activity [Avery *et al.*, 1993].

Resistance

Resistance to this group of compounds has not yet been reported. It has however been shown, that mutations in *pfmdr1* (Pgh-1) can influence sensitivity to artemisinins [Frédérich, 2002; Price *et al.*, 2004; Reed *et al.*, 2000].

1.7. Strategies to decrease malaria transmission and mortality

Due to the ever-increasing resistance of malaria parasites to currently available antimalarials, there is an urgent need for the development of novel antimalarials as well as the rational use of existing ones.

Education is an inexpensive but powerful tool that can help decrease malaria-related mortality. Educating communities about the transmission and symptoms of malaria, as well as ways to treat it, is vital. The use of vector control, such as bed nets, should be encouraged. Trape *et al.* (2002) noted that by using vector control methods 50% of the population living in malaria-infected areas could live in malaria-free areas. Underdosing and non-compliance also need to be addressed and communities must be educated about responsible drug usage in order to protect against increasing chemoprophylactic resistance [McCombie, 1996]. In addition, only drugs that are effective against parasites present in the region where the disease was contracted must be used. This will ensure optimum treatment and prevent wastage of money on ineffective drug therapy [www.who.int/tdr/diseases/malaria/diseaseinfo.htm].

Alternative drug strategies must be developed in order to safeguard the efficacy of currently available chemoprophylactics. One such method is through compounds that modulate resistance. Research into resistance reversal agents, which restore chloroquine's efficacy is of great importance due to the drug's selectivity and low cost. Another strategy is by applying combination chemotherapy with existing antimalarials in order to slow down the rate of resistance development and improve efficacy of the drugs. Uganda recently decided to replace chloroquine as its first-line treatment against malaria with a combination of sulfadoxine-pyrimethamine and chloroquine. Although chloroquine resistance is widespread in this area, the combination is more effective in lowering parasitemia and reducing fever than either of the drugs alone. It is thought that drugs, from different classes, used in combination with each other may enhance the lifetime of usage, as the chance of a strain being resistant to both drugs is smaller than that of the individual drugs [Winstanley, 2001]. The only drawbacks are that combination drugs are more

expensive and, although they may work well for the present, it is not possible to predict how long they will remain effective [Fidock *et al.*, 2004].

Research is presently being performed on developing novel antimalarials and resistance modulators, except it is very costly. Funding is a huge problem as the most affected countries do not have available money to spend on research. Malaria is also a disease that has little profit potential as it mainly affects developing countries. Pharmaceutical companies are therefore not willing to invest heavily in antimalarial research as there will be little to no return. Developments in experimental techniques and advances in knowledge, such as the sequencing of the genomes of *P. falciparum* and *Anopheles gambiae*, offer new tools to aid in research. These advances allow researchers to obtain greater insight into vital molecular mechanisms involved in drug and insecticide resistance [Carucci, D, 2004; Kanzok and Zheng, 2003]. A great deal of research has been put into developing vaccines against malaria, and with the help of genomics and proteomics a number of vaccines have already entered into the trial phase of development [Moore *et al.*, 2002]. Unfortunately it will take many years before a vaccine is available for general use [Schlagenhauf, 2003].

As mentioned previously, the control of malaria relies heavily on chemoprophylaxis. Due to the rapidly developing resistance of the parasites to currently available antimalarials, new, effective antimalarials with novel structures and modes of action need to be developed. These compounds must also be highly effective, safe and cheap in order to combat this deadly disease in developing nations.

1.8. Drug discovery

The first step in the drug discovery process is the identification of a hit compound. A hit compound possesses *in vitro* activity which could be improved via chemical modification. Preliminary structure modifications are normally performed on the hit in order to establish the molecule's pharmacophore. Repeated combinatorial chemistry and analogue synthesis potentially result in lead compounds, which hopefully progress to clinical drug candidates.

1.8.1. Hit discovery

A hit is normally selected on the basis of its *in vitro* activity and novel structure. Hits are generally new chemical entities (NCE's). NCE's are compounds with novel structures, which have been synthesized via combinatorial chemistry or isolated from a novel source, such as a natural product. Potential hit compounds are screened against a relevant and validated bioassay. These bioassays tend to be performed *in vitro*, on the causative agent of the respective disease.

There are two main approaches used to discover novel hit compounds;

Rational approach – This approach is based on the identification of a biological target and the design of ligands, which act either as agonists or antagonists. This method uses computational chemistry in order to design potential hits, which are then synthesized and tested for *in vitro* activity [Casteel, 1997].

Empirical approach – This approach screens a range of compounds derived from combinatorial libraries, historical libraries and natural sources. In the past, the focus of this approach was the development of large combinatorial libraries. These however, did not yield the expected number of NCE's. This was attributed to the scaffold or base structure's biological irrelevance. Libraries are now comprised of diverse, drug-like molecules and therefore hold more hit potential [Breinbauer *et al.*, 2002; Teague *et al.*, 1999]. Natural sources offer a great variety of novel compounds on which to base library development. Natural products offer great potential as scaffold compounds, as they are biologically validated molecules. Most importantly natural products are privileged structures – a class of compounds which can bind to various protein receptors [Evans *et al.*, 1988] and therefore libraries based on these compounds will have an enhanced likelihood of yielding hit compounds with increased efficacy than libraries based on non-privileged structures [Breinbauer *et al.*, 2002; Brohm *et al.*, 2002].

Once a hit compound has been identified, lead development occurs.

1.8.2. Lead development

Lead development focuses on developing a compound with selectively potent *in vivo* activity while optimizing absorption and bioavailability.

Leads can be classified into 3 groups; lead-like leads, high-affinity leads and drug-like leads. Table 1.8.1.1. below summarizes their respective properties.

Table 1.8.1.1.: Lead classification and optimization [Teague *et al.*, 1999].

Lead	Affinity (μ M)	Molecular weight	clogP	Optimization
Lead-like	>0.1	<350	<3	Increasing molecular weight and lipophilicity
Drug-like	>0.1	>350	>3	
High-affinity	<<0.1	>>350	<3	Reducing molecular weight and increasing lipophilicity

In general lead-like molecules offer the greatest potential for preclinical candidates and the selection of leads in this category will decrease optimization needed to be done at later stages [Teague *et al.*, 1999].

Leads can also be modified using methods that predict the biological properties of the molecules based on their chemical structures. Methods include identification of unattractive functional groups (i.e. functional groups renowned for toxicity, metabolic lability etc.), prediction of oral bioavailability as well as others [Walters *et al.*, 1999]. An example of one such method is Lipinski's 'rule of 5' [Lipinski *et al.*, 1997]. This rule predicts that poor absorption and diffusion through membranes and other cellular structures of an oral drug is more likely when;

- there are more than 5 H-bond donors (sum of OH and NH moieties)
- more than 10 H-bond acceptors (sum of Ns and Os)
- the molecular weight is over 500
- the clogP is over 5

A compound that fulfills at least three out of the four criteria adheres to Lipinski's rule.

This rule has been accepted as defining structural properties which determine a compounds absorption and oral bioavailability.

1.9. Project Background

As mentioned in the previous section, natural sources are invaluable resources of novel, biologically validated potential drugs. Medicinal plants provide approximately 80% of the world's population with disease treatment [WHO, 1995]. It is generally assumed that a plant possessing activity against a certain disease must contain active compound(s) responsible for the activity [Phillipson, 2001]. Plants are therefore major sources of biologically active privileged structures [Caniato and Puricelli, 2003], and many of the drugs used today are plant-derived [Farnsworth, 1984]. Of particular interest to this dissertation are antimalarial compounds which were isolated from plants, such as quinine and artemisinin (see section 1.6.2.). Plant-derived compounds possessing antimalarial activity tend to be secondary metabolites such as quinines, terpenes and alkaloids [Caniato and Puricelli, 2003].

Plants selected for investigation are chosen by random selection, chemotaxonomy or ethnopharmacological information [Brøgger Christensen and Kharazmi, 2001]. *Harpagophytum procumbens*, commonly known as Devil's Claw, is indigenous to Southern Africa and is traditionally used to treat a number of ailments including fevers and blood diseases. It was therefore chosen on an ethnopharmacological basis as a plant with potential antiplasmodial activity. Petroleum ether extracts of this plant possessed antiplasmodial activity, and further purification and isolation yielded compounds **1** and **2** (Figure 1.9.1.). Both compounds **1** and **2** were found to possess significant selective antiplasmodial activity, and due to their common novel structures were used as hits for further structure activity relationship (SAR) studies. The low yields at which **1** and **2** were isolated prompted the identification of totarol, a commercially available analogue, for chemical modification purposes [Clarkson *et al.*, 2003a, b].

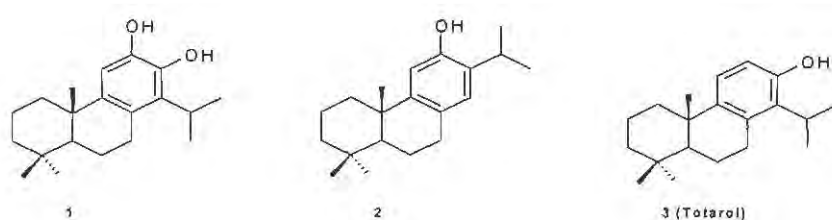


Figure 1.9.1.: Compounds **1** and **2** isolated from *Harpagophytum procumbens* and totarol (**3**) [Clarkson *et al.*, 2003a].

The first set of derivatives synthesized were a range of β -amino alcohol derivatives (Figure 1.9.2.). The β -amino alcohol moiety is a common structural feature in a number of antimalarials, such as quinine and mefloquine (see Figure 1.6.2.2.1.) where it forms part of a pharmacophore consisting of an aromatic and an amino alcohol moiety separated by 2-3 carbon atoms. It was thought that combining the privileged naturally derived hit totarol with a potentially active amino alcohol moiety would yield derivatives with improved antiparasmodial activity.

Comparison of the results obtained show that all the derivatives synthesized showed improved activity, and in some cases improved selectivity, against the intraerythrocytic stage of *P. falciparum* [Clarkson *et al.*, 2003b]. This project is a continuation of these studies.

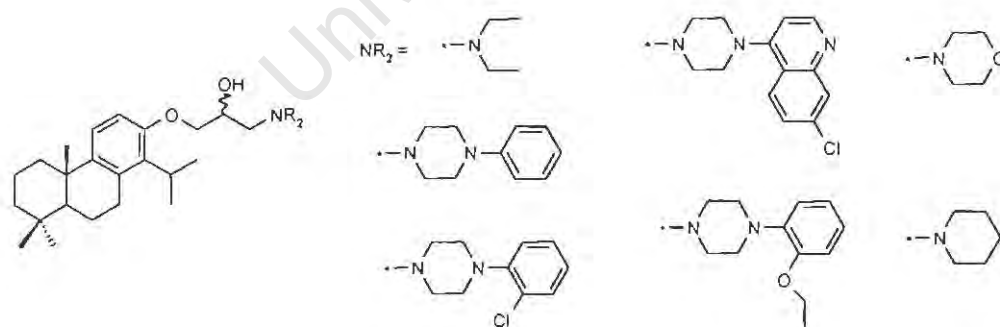


Figure 1.9.2.: β -amino alcohol derivatives of totarol synthesized by Clarkson *et al.* (2003b).

1.10. Tatarol

Tatarol (**3**) is a commercially available compound, and a number of methods describing its synthesis have been reported [Bendall and Cambie, 1995]. It is also a naturally occurring phenolic diterpenoid, which has been found in a number of plants [Bendall and Cambie, 1995]. It was first isolated in 1910 from *Podocarpus totara* [Bendall *et al.*, 1994], and was the first compound to be discovered with the chemical formula of $C_{20}H_{30}O$. Tatarol was named as such as it was believed to possess a tertiary alcoholic group due to the slow rate at which it underwent acetylation. Its structure (Figure 1.10.1.) was first identified by Short and Wang (1951).

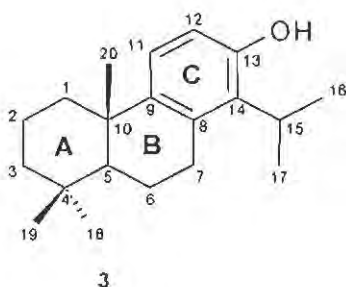


Figure 1.10.1.: Structure of Tatarol [Short and Wang, 1951].

Tatarol's chemical name is totara-8,11,13-trien-13-ol and its nomenclature is based on the parent compound totarane [Bendall *et al.*, 1994]. The phenol group is sterically hindered by the isopropyl group, as determined by the IR spectrum and this accounts for tatarol's slow rate of acetylation. Steric shielding is present which prevents intermolecular hydrogen bonding [Short and Wang, 1951] and thus decreases tatarol's apparent polarity [Bendall *et al.*, 1994]. The positioning of substituents, such as the attachment of the isopropyl group on C-14, results in tatarol's unique chemistry.

Tatarol and derivatives were shown to possess antibacterial activity against Gram-positive bacteria only [Evans *et al.*, 1999; 2000]. This may be due to these lipophilic compounds being trapped in the cell membrane lipids of Gram-negative bacteria [Lien *et al.*, 1968], which contain a greater percentage of lipids. Derivatives with modifications at rings A and B, C-12 and O-13, showed varying degrees of antibacterial activity although none of the

derivatives were found to have greater antibacterial activity than that of their parent compound totarol [Evans *et al.*, 2000]. Structure-activity relationship studies performed on totarol, have demonstrated that the phenolic moiety at C-13 is essential for its antibacterial activity [Evans and Furneaux, 2000]. (+)-Totarol has also been reported to be active against *Mycobacterium tuberculosis* [Constantine *et al.*, 2001]. It has been suggested that totarol's site of action may be the cell membrane [Haraguchi *et al.*, 1996], or that these lipophilic phenolic compounds (totarol and derivatives) may act either by interfering with cell wall biosynthesis or by interfering with oxidative phosphorylation via the uncoupling effect. The uncoupling effect disables ATP synthesis by preventing the formation of the electrochemical potential difference across the cell membrane. The uncoupling effect of totarol was investigated, but it was found that it was unlikely to be responsible for the compounds' antibacterial activity [Evans *et al.*, 2000].

Totarol has also been found to protect against oxidative stress and has also been shown to exert hypercholesterolemic activity in rats. It is thought that totarol acts by inhibiting cholesterol absorption from the intestine as well as stimulating cholesterol breakdown and/or excretion [Enomoto *et al.*, 1977].

1.11. Project Focus

The focus of this project is to synthesize and identify possible bioactiphore(s)* of the antimalarial hit compound totarol. Hit and lead development tools, as discussed above, are therefore extremely relevant at this stage of research in order to maximize both the results and value of the research.

* A bioactiphore is the minimum structural requirement a molecule must contain in order to exhibit *in vitro* or *in vivo* activity. In the case of a pharmacophore, the receptor(s) is known and therefore the moieties needed for optimum binding of the receptor(s) constitute the pharmacophore.

Chapter 2

Project Objectives and Rationale

2.1. Project Objective

The overall objective of this project is to develop promising potential antiplasmodial lead compounds through chemical modification and SAR (structure-activity relationship) studies of a range of totarol derivatives.

2.2. Specific Aims

1. Synthesize a number of totarol derivatives and analogues using traditional medicinal chemistry techniques.
2. Characterization of derivatives using analytical and spectroscopic techniques including:
 - Polarimetry
 - Melting point
 - Elemental analysis / HPLC
 - Mass spectrometry
 - Infrared spectroscopy (IR)
 - Nuclear Magnetic Resonance (NMR) - ^1H , ^{13}C , 2D (COSY, HMBC, HSQC)
3. Evaluation of the *in vitro* antiplasmodial activity of the derivatives against a chloroquine sensitive (D10) strain of *P. falciparum*.
4. Evaluation of the *in vitro* antiplasmodial activity of the derivatives, showing activity greater than that of totarol in the D10 strain, in a chloroquine resistant K1 strain of *P. falciparum*.
5. Cytotoxic evaluation of the most active derivatives using a mammalian Chinese Hamster Ovarian (CHO) cell line.
6. Investigation into the ability of structurally promising derivatives to reverse chloroquine resistance in a chloroquine resistant (K1) strain of *P. falciparum*.

7. Evaluation of selected analogues' and derivatives' effect on erythrocytic invasion in a chloroquine sensitive (D10) strain of *P. falciparum*.

2.3. Chemical modifications on totarol

2.3.1. Summary of target compounds

The following totarol derivatives and analogues (Figure 2.3.1.1.), except phenolic precursors, will be synthesized via methods described in chapter 3.

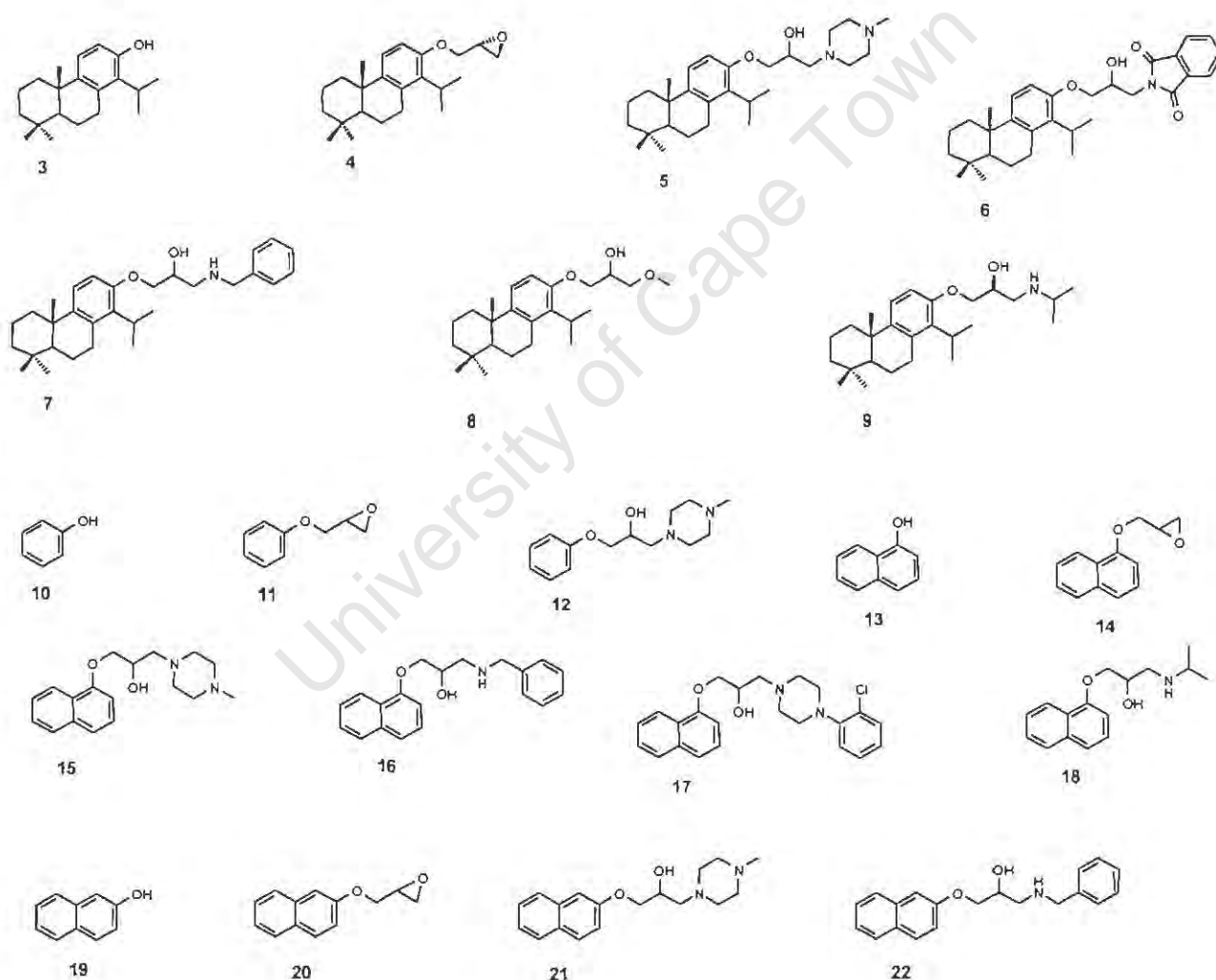


Figure 2.3.1.1.: Parent compounds, totarol (3), phenol (10), 1-naphthol (13) and 2-naphthol (19) as well as target compounds (5-9, 12, 15-18, 21 and 22) that will be synthesized.

2.3.2. Rationale for synthesis of target compounds

2.3.2.1. Investigation of the SAR within the β -amino alcohol series (Compounds **5**, **6**, **7**, **9**)

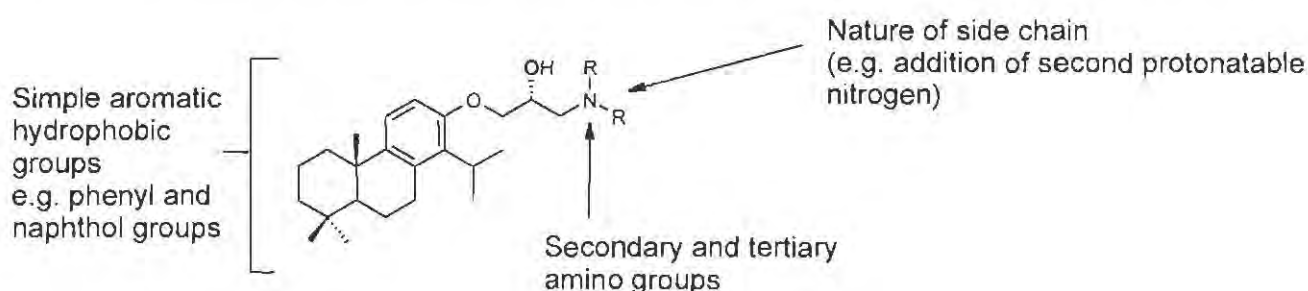


Figure 2.3.2.1.1.: Summary of modifications

Nature of the β -amino group

This study is aimed at exploring the effect of substitution on the β -amino group by comparing secondary and tertiary β -amino substitution. In this regard a secondary β -amino alcohol **7** and a tertiary β -amino alcohols **5** and **6** will be synthesized. The addition of a second protonatable nitrogen such as in **5** is predicted to increase the compound's passage across the cell membrane and accumulation in the parasites acidic food vacuole via pH trapping. Compounds which are weakly basic are likely to become trapped in acidic compartments due to the existing transmembrane proton gradients. The unprotonated base form of the compound readily crosses the membrane into the acidic compartment where it is protonated. This protonated form cannot leave the acidic compartment as it cannot cross the membrane. pH trapping is thought to account in part for the high levels of chloroquine which accumulates in the acidic food vacuole of the malaria parasite [Yayon *et al.*, 1984]. The activity displayed by **5** will be compared with the activity reported for a totarol derivative with one protonatable nitrogen [Clarkson *et al.*, 2003b].

Substitution of totarol backbone

Substitution of the totarol diterpenoid-like hydrophobic backbone will allow conclusions to be drawn about its role in the activity displayed by the totarol derivatives. The totarol backbone will be replaced by simple aromatic groups such as phenyl and naphthyl.

Resistance reversal ability

In general, compounds which act to reverse chloroquine resistance have similar structural features, such as; lipid solubility, two planar aromatic rings, a cationic charge and a tertiary nitrogen [Beck, 1990; Zamora *et al.*, 1988]. Totarol derivatives with similar features will be tested for resistance reversal ability.

Effect and comparison of compounds **9** and **18** on erythrocytic invasion

Haldar *et al.*, (2003) proposed that erythrocytic G protein-coupled receptor signaling, involving the β 2-adrenergic receptor, may be necessary for parasitic invasion of the erythrocyte. They demonstrated that β -blockers (β -adrenergic receptor antagonists) inhibit *in vitro* *P. falciparum* infection of erythrocytes and decrease parasitaemia *in vivo* in mice infected with *Plasmodium berghei*.

β -Blockers are commonly used to treat hypertension and tend to possess at least 1 aromatic ring structure which is attached to an alkyl side chain possessing a secondary hydroxyl and amine moiety, as well as one or more chiral centers. At least one of the chiral centers in the alkyl side chain is directly attached to the hydroxyl group [Mehvar and Brocks, 2001].

Racemic propranolol (β -blocker) was shown by Haldar *et al.*, (2003) to inhibit infection, although the antagonistic behaviour was stereospecific, as demonstrated by the (+) inactive enantiomer. It will therefore be synthesized in racemic form (**18**) and its effect on erythrocytic invasion will be compared with that of its corresponding totarol derivative (**9**).

Chapter 3

Synthesis and Characterization of Totarol Derivatives and Analogues

3.1. General mechanisms

Figure 3.1.1. summarizes the synthesis of the β -amino alcohol totarol derivatives and analogues.

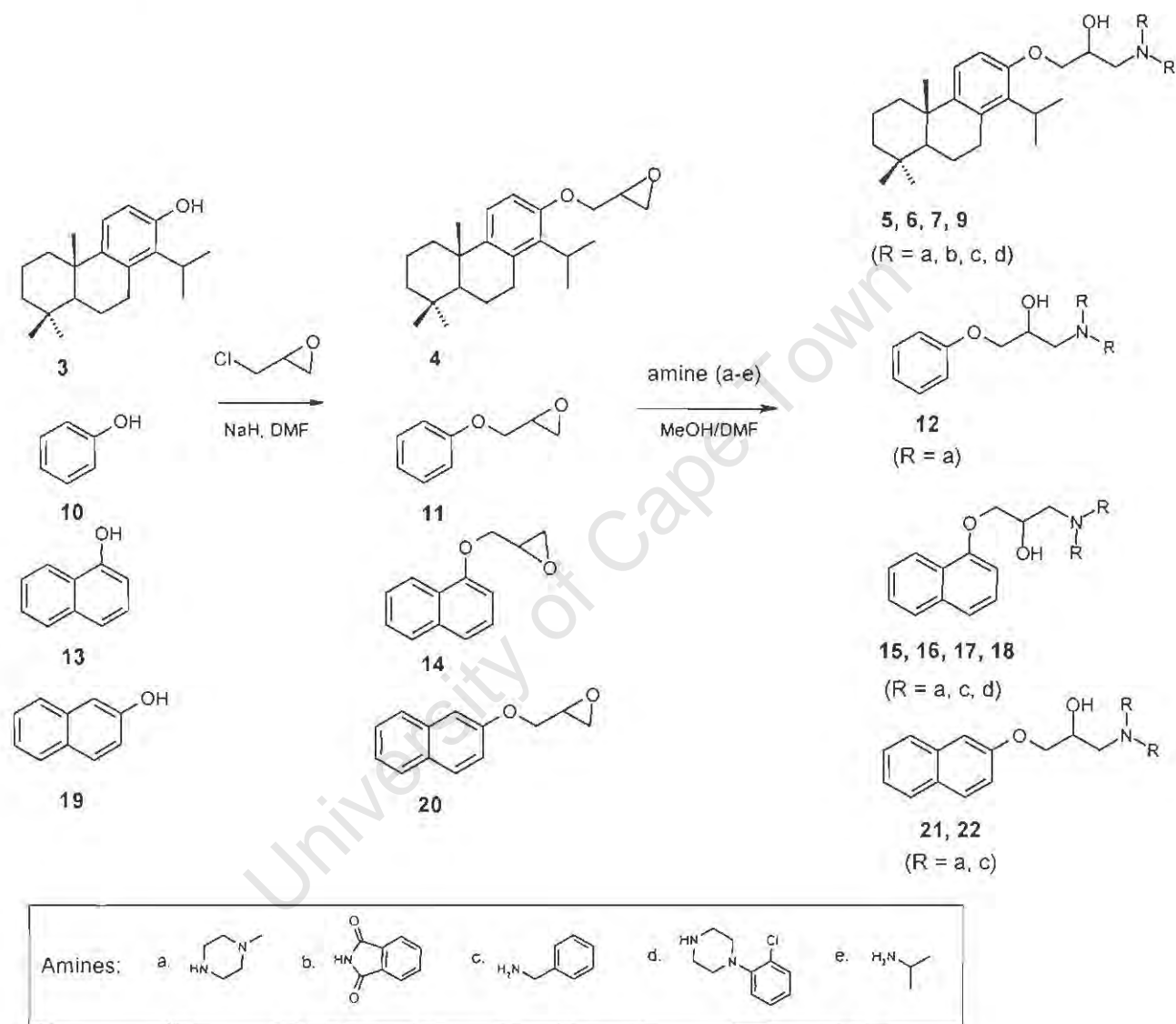


Figure 3.1.1.: Scheme showing a summary of β -amino alcohol synthesis.

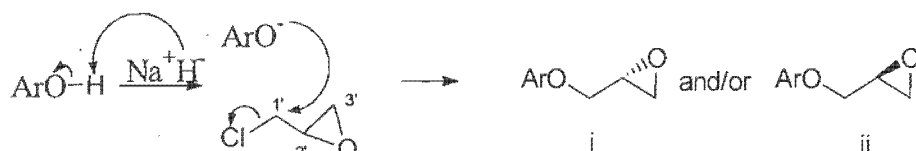
The starting materials (totarol, **3**, phenol, **10**, 1-naphthol, **13** and 2-naphthol, **19**) were dissolved in DMF, deprotonated by sodium hydride (NaH) and alkylated with epichlorohydrin, resulting in the formation of the epoxide intermediates (**4**, **11**, **14** and **20**). The epoxide intermediate was then dissolved in methanol, except in the formation of **6**

where a catalytic amount of potassium phthalimide and DMF (solvent) were used, and an excess of the amine was added, resulting in the formation of the respective β -amino alcohol derivatives with percentage yields in the range of 29-93% [Lawrence and Bushell, 2001; Williams *et al.*, 1985].

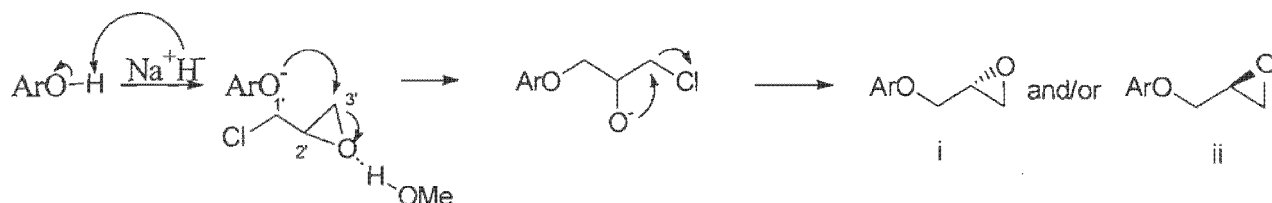
Epoxide intermediate formation

Figure 3.1.1. below depicts the mechanism by which the epoxide intermediates **4**, **11**, **14** and **20** are formed. In the presence of the strong base, NaH, totarol is converted to a phenoxide anion. There are three electrophilic carbons in epichlorohydrin (carbons 1', 2' and 3') at which this anion could potentially attack (Figure 3.1.1.). The regiochemistry of the reaction is however markedly solvent-dependant. The use of an aprotic solvent, such as DMF, favours the reaction shown in scheme A, in which the carbon-halide bond is most likely to be attacked by the phenoxide nucleophile. In an alcoholic solvent, hydrogen bonding weakens the carbon-oxygen bond favouring attack at carbon 3' rather than carbon 2' on steric grounds. An intramolecular SN2 reaction then occurs, resulting in displacement of Cl and formation of epoxides i and ii (scheme B). In the presence of an acid, the oxygen of the epoxide is protonated or complexed to a Lewis acid and attack at carbon 2' is favoured in order to stabilize the developing secondary positive charge (scheme C) [Cruickshank and Fishman, 1969]. As DMF was used as a solvent (aprotic), nucleophilic attack occurs at carbon 3', resulting in an opening up of the epoxide.

Scheme A



Scheme B



Scheme C

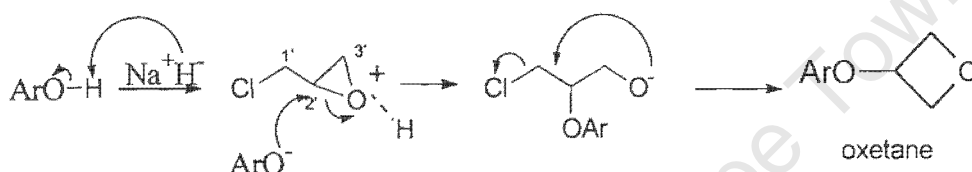
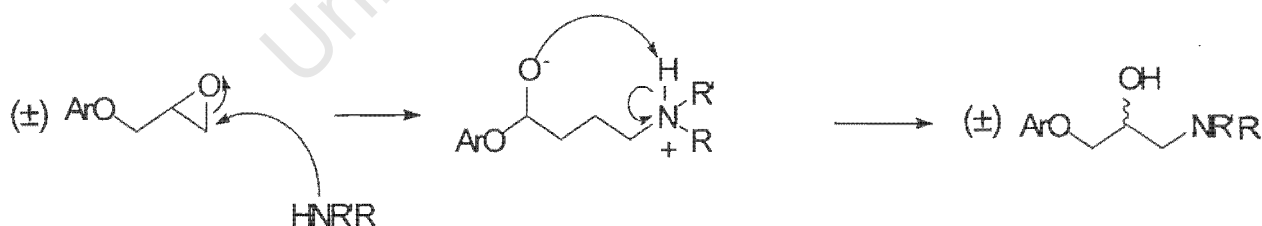


Figure 3.1.2.: Mechanism of epoxide formation.

 β -Amino alcohol formation

Nucleophilic attack of the amine at the less sterically hindered carbon of the epoxide intermediate results in ring opening to generate an intermediate alkoxide anion, which after proton transfer results in the product β -amino alcohol (Figure 3.1.2.).



R, R' = alkyl or aryl

Figure 3.1.3.: β -Amino alcohol formation

In the case of tatarol β -amino alcohols, enantiomeric resolution of the racemic epichlorohydrin due to the enantiomerically pure starting material, tatarol, results in the

formation of one diastereomer for the epoxide intermediate and as such for the β -amino alcohol products.

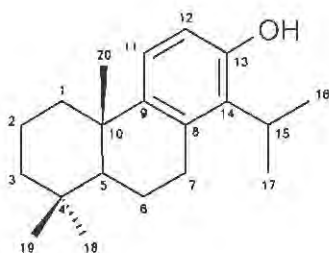
3.2. Spectroscopic Indicators and Analyses

Only key spectroscopic signals from ^1H NMR will be discussed, as it is the key method used to evaluate target compounds. Selected spectra are shown in appendix 3.

The resonances are described as they appear (chapter 4). Possible explanations are given below. It should be noted however, that resonances may appear as other multiplets other than those expected due to small coupling constants and poor resolution (e.g. a double doublet with small coupling constants may appear as a triplet).

Starting compounds **3**, **10**, **13** and **19**

Totarol, 3



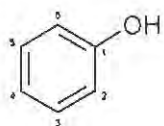
Complete ^1H and ^{13}C data has been reported for totarol [Ying and Kubo, 1991]. ^1H , ^{13}C , COSY, HMBC and HSQC experiments were performed to confirm the identity of the compound and to aid in the ^1H and ^{13}C assignment of the derivatives.

The hydroxyl group was identified at δ 4.42 using a D_2O wash. The H-11 proton (δ 7.00) resonates as a doublet ($J = 8.8$). H-11 was found to couple with a doublet (δ 6.51, $J = 8.8$) in the COSY spectrum, which was identified as H-12. The mesomeric effect of the hydroxyl group at H-13 causes H-12 to resonate upfield to H-11. Coupling of H-15 (sextet at δ 3.30) to two doublets (integrating for 3 protons) identified H-16 and H-17 (COSY). The methyl groups at H-16 and H-17 are diastereotopic. The H-1 α appears as a multiplet due to

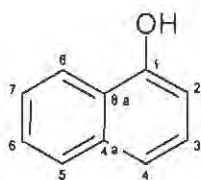
coupling with H-1 β and H-2 β , at a δ of 1.17-1.24. H-1 α does not couple with H-2 α as the two protons are orthogonal to each other. The coupling of H-1 β with H-1 α (geminal) results in the splitting of the signal into a broad doublet. H-1 β is also orthogonal to H-2 α and H-2 β and therefore does not couple to these protons. H-2 α and H-2 β couple with 2 neighboring fortuitously equivalent hydrogens, resulting in splitting of both signals into double triplets. H-3 α forms a multiplet by coupling to H-2 β and H-3 β . It does not couple to H-2 α , as the two are orthogonal. H-3 β couples to H-2 α (vicinal) and H-3 α (geminal) resulting in a broad doublet. H-5 is orthogonal to H-6 α , but couples with H-6 β and its signal appears as a multiplet. Coupling of H-6 α with H-6 β and H-7 α splits its signal into a double doublet. H-6 β is visible as a multiplet between δ 1.55 –1.70. H-7 α is also a multiplet, coupling with H-6 α and H-7 β . H-6 β and 7 α are orthogonal, and therefore do not couple with each other. H-7 β couples with H-6 β and 7 α , resulting in the formation of a double doublet. HSQC also linked H-7 α and H-7 β to the same carbon (C-7). The methyl groups at positions 18, 19 and 20 all appear as singlets (with an integration of 3), as they do not have neighboring protons with which to couple.

HMBC and HSQC were used to aid in the assignment of the peaks in the ^{13}C spectrum.

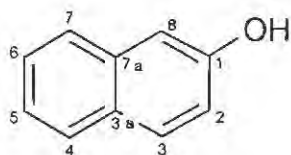
Phenol, 10



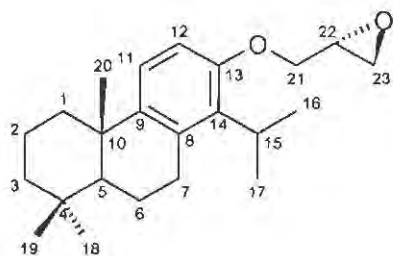
Hydrogens at positions 2 and 6 are chemically equivalent and the most shielded due to their *ortho* positioning with respect to the hydroxyl group. These two protons therefore appear the furthest upfield and resonate as double doublets. H-4 appears as a triple triplet due to large, *ortho* couplings with H-3 and H-5 and smaller *meta* couplings with H-2 and H-6. H-3 and H-5 also resonate as a triple triplet, and are most deshielded therefore appearing downfield. The position of the hydroxyl group at δ 4.85 was established by a D_2O wash.

1-Naphthol, 13

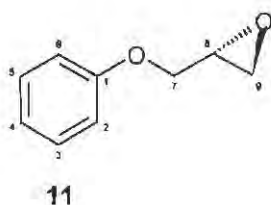
Assignments were established based on the literature as well as resonance structures. H-2 will resonate as a double doublet, as it is *ortho* coupled to H-3 and *meta* coupled to H-4. H-4 is a triplet due to coupling with H-2 and H-3. H-5 is a doublet due to coupling with H-6. H-7 and H-8 are multiplets. H-7 appears as a multiplet due to through space deshielding due to the hydroxyl group at C-1. H-3 and H-6 are multiplets, although H-6 is the most deshielded as it is furthest from the hydroxyl moiety.

2-Naphthol, 19

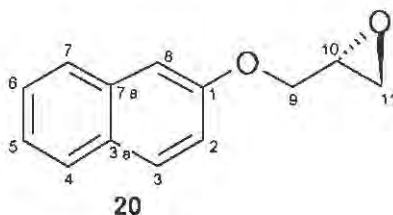
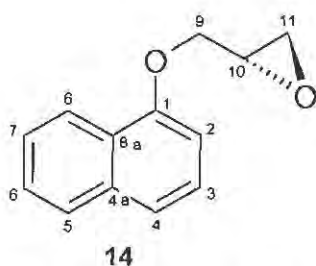
Assignments were established based on the literature as well as resonance structures. H-4 and H-6 are the most deshielded due to ring resonance. They therefore appear furthest downfield and couple to two chemically equivalent neighbours, H-5 (*ortho*) and H-7 (*para*) and H-5 and H-7 (*ortho*) respectively. H-3 and H-8 resonate as doublets due to coupling with H-2. Protons at positions 5 and 7 resonate as double triplets due to coupling with protons in the same ring. H-2 is the most shielded, and therefore resonates furthest upfield. It appears as a double doublet due to coupling with H-3 and H-8.

Intermediates: Compounds 4, 11, 14 and 20

Compound **4**: H-21a and b were both present as triplets due to geminal and vicinal coupling (H-22). These two protons are diastereotopic and therefore interchangeable. H-22 couples with H-21a, H-21b and H-23 α resulting in a quintet. H-22 and H-23 β are orthogonal and therefore do not couple. The protons at position 23 are diastereotopic and hence both are seen as double double doublets due to coupling with H-23 (α or β) and H-22.



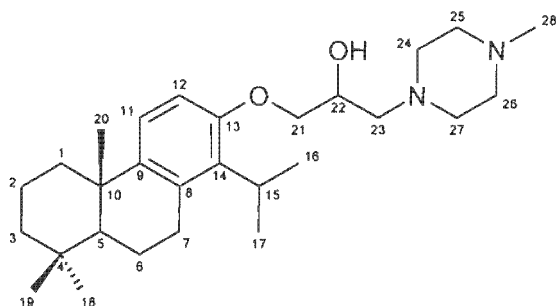
Compound **11**: H-7a and b appear as double doublets, due to coupling with each other and with H-8. H-8 and H-9 α appear as a multiplet. H-9 β couples with H-9 α and H-8 and therefore appears as a double doublet.



Compounds **14** and **20**: H-9a and b and H-11 α and β appear as double doublets, due to geminal coupling and vicinal coupling with H-10.

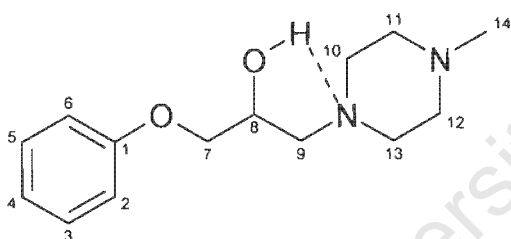
Derivatives

Compounds 5, 12, 15 and 21

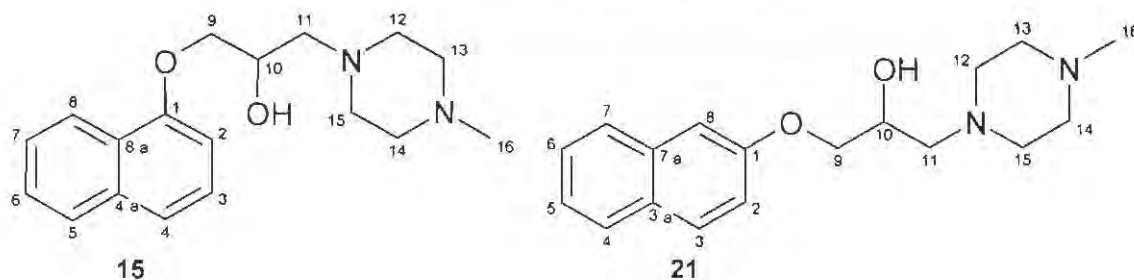


Compound 5: The protons at C-21 are diastereotopic, and each is visible as a double doublet due to geminal coupling with one another and vicinal coupling with H-22.

Protons at positions 24, 25, 26 and 27 appear as a multiplet. H-28 does not have any neighbouring protons to couple to and therefore appears as a singlet.

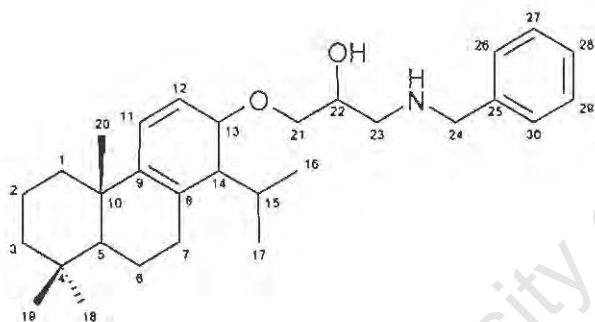


Compound 12: The protons at C-7 are chemically equivalent and therefore are only visible as a doublet (integrating for 2), due to coupling with H-8. Protons at C-9 are chemically equivalent and appear as a broad doublet due to coupling with H-8. Protons at C-10 and C-13 appear downfield to those at C-11 and C-12, possibly due to intramolecular hydrogen-bonding as shown.



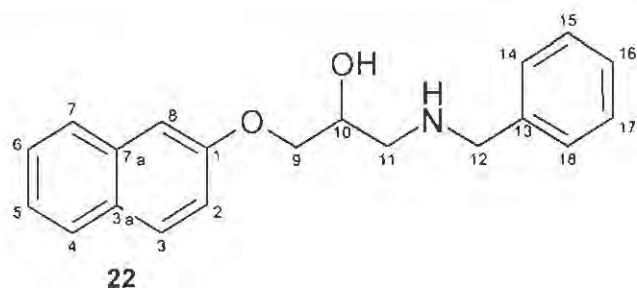
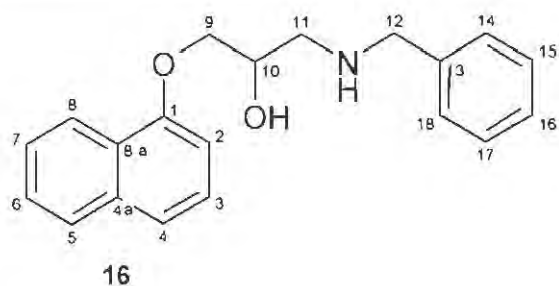
Compounds **15** and **21**: Protons at C-13 and C-14 are chemically equivalent and appear as a broad singlet upfield to H-12 and H-15, as they are further away from the hydroxyl moiety and are therefore more shielded. Protons H-12 and H-15 are chemically equivalent and appear as a broad singlets in **15** and **21**.

Compound **7**, **16** and **22**:



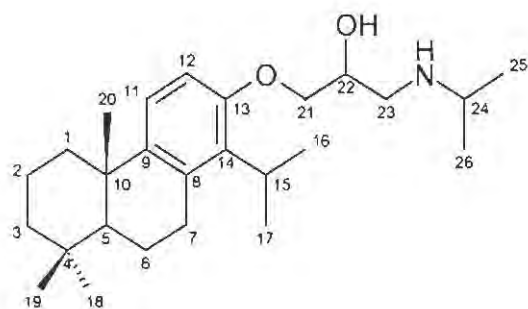
Compound **7**: H-21 α and β are interchangeable and both are visible as double doublets due to geminal and vicinal (H-22) coupling. H-22 appears as a multiplet. H-23 appears as a multiplet, due to coupling with H-22. H-24, NH and OH appear as singlets. Protons at positions 26-30 all have similar chemical shifts and therefore appear as a multiplet in the region 7.26-7.37.

The resonances of the OH and NH moieties were verified by a D₂O wash.

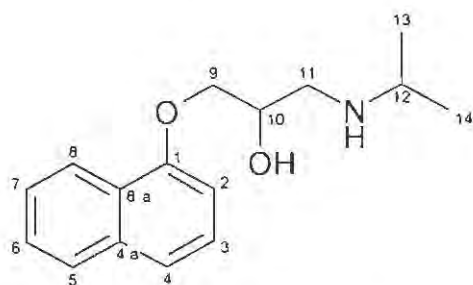


Compounds 16 and 22: All protons appeared at similar chemical shifts to those seen in compound 7. Their multiplicities were not constant, although this is probably due to inter-molecule variation and chemical nature.

Compounds 9 and 18:



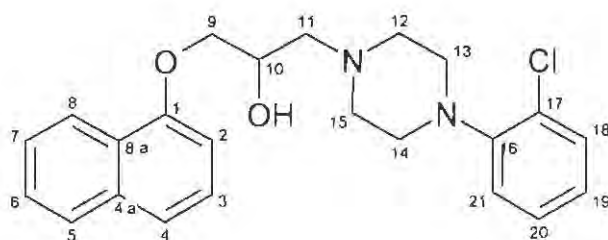
Compound 9: H-22 is the most deshielded out of all the hydrogens in the side chain. H-21a and b each couple with three neighbours (each other, H-22 and H-23a or b). H-24 has been designated as a broad singlet although, the peak has slight splittings on either side. It does couple with H-25 and H-26, as these protons do appear as doublets.



Compound 18: The NH and OH moieties were apparent due to their chemical shifts and D₂O wash. H-9b appears as a double doublet due to coupling with H-10 and H-9a. H-11a

and b both appear as double doublets due to geminal and vicinal coupling. H-12 appears as a multiplet due to coupling with H-13 and H-14. H-13 and H-14 are chemically equivalent and therefore appear as a doublet due to coupling with H-12.

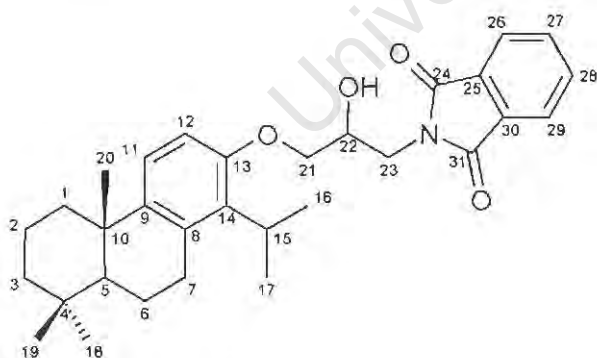
Compound 17



H-10 appears as a quintet, due to equal splitting by four neighbours (a and b protons of H-9 and H-11).

Chlorophenyl moiety: H-18 is the most deshielded, because of the inductive effect of the Cl, and appears as a doublet due to coupling with H-19. H-20 is *para* to C-17 and is therefore also fairly deshielded. It appears as a double doublet, due to unequal couplings with H-19 and H-21. H-21 appears as a doublet due to splitting by H-20.

Compound 6



H-26 and H-29 are chemically equivalent and the most deshielded protons due to their position in an aromatic ring and proximity to the carbonyl groups. They appear as double doublets due to *ortho* and *meta* coupling with H-27 and H-28. H-27 and H-28 are also

equivalent and relatively shielded, due to their *meta* positions. They appear as double doublets due to *ortho* and *meta* coupling with H-26 and H-29.

Mass spectra and elemental analysis/HPLC indicated the target amino alcohols were correct and pure respectively. Compounds' calculated molecular weights are shown in appendix 1 for comparative purposes. All amino alcohols also displayed an OH band at 3000-3700 cm^{-1} in the IR spectrum.

University of Cape Town

Chapter 4

Materials and Methods

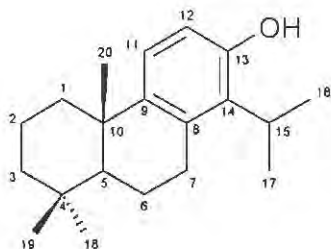
4.1. General

Totarol was purchased from Sigma-Aldrich, South Africa, 1- and 2- naphthol from Merck and Judex respectively and epichlorohydrin was purchased from British Drug House (BDH). The remaining chemicals were obtained from Sigma-Aldrich, South Africa. All reactions were monitored via TLC, which was carried out on Merck silica 60 F₂₅₄ coated aluminium sheets. Preparative TLC was performed on Merck silica 60 F₂₅₄ coated plates and column chromatography was performed on Merck Kieselgel silica gel 60. Micro (elemental) analysis on all target compounds, except compounds **6**, **7** and **17**, was performed on a Fisons EA 1108 CHNS-O instrument. Purity of compounds **6**, **7** and **17** was confirmed via HPLC on a Waters 996 HPLC and photodiode array detector. The HPLC spectra were run on a C18 column with an acetonitrile/water gradient at wavelengths ranging between 190-400nm. IR spectra of all compounds, except **7** (1mg/ml solution in DCM), were recorded using KBr disks on a Thermo Mattson FTIR spectrometer in the range of 4000-500 cm⁻¹. Low resolution mass spectra (LREIMS) of compounds **4**, **6-8** were obtained on an VG micromass 16 F spectrometer at 70eV while those for compounds **5**, **9**, **12**, **15-18**, **21** and **22** were obtained on an Applied Biosystems API 2000. ¹H, ¹³C, COSY, HSQC and HMBC NMR spectra were recorded on Varian 300 MHz or 400 MHz instruments with TMS as a reference. Chemical shifts are reported in parts per million and coupling constants in hertz (Hz).

Selected NMR and IR spectra are shown in appendix 2.

Starting compounds 3, 10, 13 and 19

All starting compounds were verified via ^1H and ^{13}C NMR. The assignments are shown below.

Totarol, 3

^1H NMR δ_{H} (400 MHz; CDCl_3) 7.00 (d, $J = 8.8$, H-11), 6.51 (d, $J = 8.8$, H-12), 4.42 (s, OH), 3.30 (sept, $J = 7.2$, H-15), 2.94 (dd, $J = 17, 6.2$, H-7 β), 2.75 (m, H-7 α), 2.23 (br d, $J = 11.6$, H-1 β), 1.92 (dd, $J = 7.7, 1.6$, H-6 α), 1.73 (dt, $J = 13.7, 3.3$, H-2 β), 1.55–1.70 (m, H-6 β), 1.49 (dt, $J = 13.2, 3.2$, H-2 α), 1.47 (br d, $J = 13.2$, H-3 β), 1.36 (d, $J = 7.1$, H-17), 1.34 (d, $J = 7.1$, H-16), 1.18 (s, H-20), 1.17–1.30 (m, H-1 α , H-3 α , H-5), 0.95 (s, H-18), 0.92 (s, H-19). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 152.0 (C-13), 131.0 (C-14), 123.0 (C-11), 114.3 (C-12), 49.6 (C-5), 41.6 (C-3), 39.6 (C-1), 37.7 (C-10), 33.3 (C-4), 33.2 (C-18), 28.7 (C-7), 27.1 (C-15), 25.4 (C-20), 21.6 (C-19), 20.3 (C-16, C-17), 19.5 (C-2), 19.4 (C-6).

Optical rotation of totarol

The angle of rotation was measured on a Perkin-Elmer 141 Polarimeter.

The optical rotation of a compound is calculated via the following formula [Clayden *et al.*, 2001]:

$$[\alpha]_D^T = \alpha / cl$$

where D indicates the wavelength of 589nm,

T indicates the temperature ($^{\circ}\text{C}$),

α is the observed angle of rotation (measured 3 times and averaged),

c is the concentration (g/ml) and

l is the path length (dm).

In this case;

$$\alpha = +0.0415$$

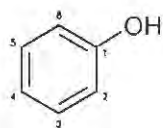
$$c = 0.001 \text{ g/ml}$$

$$l = 0.89 \text{ dm}$$

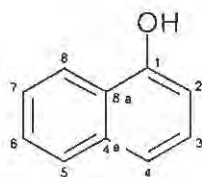
$$\therefore [\alpha]_{\text{D}}^{20} = +46.629^{\circ}$$

The literature value of (+)-totalol is reported as $[\alpha]_{\text{D}} = +41.5^{\circ}$ [Matsumoto and Suetsugu, 1979].

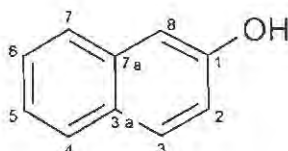
Phenol, 10



^1H NMR δ_{H} (400 MHz; CDCl_3) 7.25 (tt, 7.5, 1.8, H-3, H-5), 6.94 (tt, $J = 7.8, 1.3$, H-4), 6.82-6.86 (dd, $J = 7.8, 1.2$, H-2, H-6), 4.85 (s, OH). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 155.5 (C-1), 129.7 (C-3, C-5), 120.8 (C-4), 115.3 (C-2, C-6).

1-Naphthol, 13

^1H NMR δ_{H} (400 MHz; CDCl_3) 8.20 (m, H-6), 7.83 (m, H-3), 7.50 (m, H-7, H-8), 7.46 (d, J = 8.4, H-5), 7.32 (t, J = 7.6, H-4), 6.82 (dd, J = 7.6, 1.2, H-2). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 151.6 (C-1), 135.0 (C-4a), 127.9 (C-5), 126.7 (C-6), 126.0 (C-7), 125.5 (C-3), 124.6 (C-8a), 121.7 (C-8), 120.9 (C-4), 108.87 (C-2).

2-Naphthol, 19

^1H NMR δ_{H} (400 MHz; CDCl_3) 7.76 (t, J = 8.0, H-4, H-6), 7.67 (d, J = 8.4, H-3), 7.43 (dt, J = 8.0, 1.3, H-7), 7.33 (dt, J = 7.6, 1.2, H-5), 7.15 (d, J = 2.4, H-8), 7.10 (dd, J = 8.8, 2.4, H-2). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 153.3 (C-1), 134.6 (C-7a), 129.8 (C-3), 129.0 (C-3a), 127.8 (C-4), 126.5 (C-6), 126.4 (C-7), 123.6 (C-5), 117.7 (C-2), 109.5 (C-8).

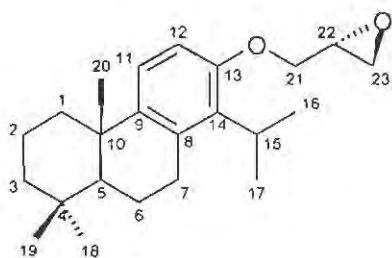
General Experimental Protocol for Synthesis of β -amino alcohols

Synthesis of epoxide intermediates **4**, **11**, **14** and **20**

General Procedure

Sodium hydride (1.5 eq.) was added to a solution of the respective starting compounds **3**, **10**, **13** or **19** (1 eq.) in DMF at 0°C under nitrogen. The reaction mixture was raised to 25°C (or 50°C in the case of phenyl epoxide formation) and epichlorohydrin (5 eq.) was added dropwise over 2 minutes. The reactions were complete after 12 hours as confirmed via TLC. The mixture was diluted with water (100ml), extracted with EtOAc (3 X 100ml) and dried over MgSO₄. Excess epichlorohydrin and EtOAc was removed in vacuo to yield an oil which was purified on a silica gel column.

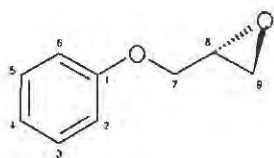
Compound **4**:



11.1g (3.88 mmol) of totarol (**3**), 0.14g (5.82 mmol) NaH and 1.52 (19.41 mmol) epichlorohydrin used, resulting oil purified via silica gel chromatography, eluting with Et₂O/Hex (1:12), R_f = 0.56, giving an isolated yield of 84% (1.12 g). ¹H NMR δ_H (400 MHz; CDCl₃) 7.07 (d, J = 8.8, H-11), 6.67 (d, J = 8.8, H-12), 4.16 (t, J = 10.9, 3.0, H-21a), 4.14 (t, J = 10.9, 3.0, H-21b), 3.98 (ddd, J = 10.8, 5.2, 2.4, H-23 α), 3.95 (ddd, J = 10.8, 5.2, 2.4, H-23 β), 3.36 (m, H-22), 3.3 (sept, J = 7.2, H-15), 2.95 (dd, J = 16.9, 6.0, H-7 β), 2.75 (m, H-7 α), 2.24 (br d, J = 11.6, H-1 β), 1.91 (dd, J = 7.9, 1.9, H-6 α), 1.73 (dt, J = 13.4, 3.9, H-2 β), 1.55 – 1.72 (m, H-6 β), 1.49 (dt, J = 13.2, 3.2, H-2 α), 1.47 (br d, J = 13.2, H-3 β), 1.36 (d, J = 7.1, H-17), 1.34 (d, J = 7.1, H-16), 1.18 (s, H-20), 1.16-1.30 (m, H-1 α , H-3 α , H-5), 0.95 (s, H-18), 0.92 (s, H-19). ¹³C NMR δ_C (100.6 MHz; CDCl₃) 153.3 (C-13), 133.5 (C-14),

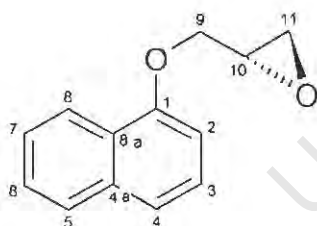
122.7 (C-11), 110.1 (C-12), 68.7 (C-21), 50.3 (C-22), 49.5 (C-5), 44.7 (C-23), 41.5 (C-3), 39.6 (C-1), 37.7 (C-10), 33.1 (C-4), 33.0 (C-18), 28.7 (C-7), 27.5 (C-15), 25.1 (C-20), 21.5 (C-19), 24.4 (C-16, C-17), 19.4 (C-2), 19.3 (C-6).

Compound 11:

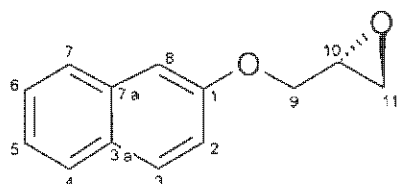


Reaction was performed at 50°C. 1.07g (11.39 mmol) of phenol (**10**), 0.82 (34.17 mmol) NaH and 4.45ml (56.95 mmol) epichlorohydrin used, resulting oil purified via silica gel chromatography, eluting with EtOAc/Hex (1:12), R_f = 0.48, giving an isolated yield of 88% (1.50 g). ^1H NMR δ_{H} (400 MHz; CDCl_3) 4.21 (dd, J = 14.8, 4.4, H-7a), 3.98 (dd, J = 11.1, 5.4, H-7b), 3.33-3.38 (m, H-8), 2.90 (m, H-9 α), 2.75 (dd, J = 5.1, 3.3, H-9 β). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 69.0 (C-7), 50.2 (C-8), 44.7 (C-9).

Compound 14:



1.02g (7.08 mmol) of 1-naphthol (**13**), 0.25g (10.5 mmol) NaH and 2.74ml (35 mmol) of epichlorohydrin used, resulting oil purified via silica gel chromatography, eluting with Et_2O /Hex (3:7), R_f = 0.40, giving an isolated yield of 76% (1.08 g). ^1H NMR δ_{H} (400 MHz; CDCl_3) 4.40 (dd, J = 11.2, 3.2, H-9a), 4.17 (dd, J = 11.0, 5.4, H-9b), 3.48-3.51 (m, H-10), 2.97 (dd, J = 5.0, 4.2, H-11 α), 2.85 (dd, J = 5.0, 2.6, H-11 β). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 69.0 (C-9), 50.2 (C-10), 44.7 (C-11).

Compound **20**:

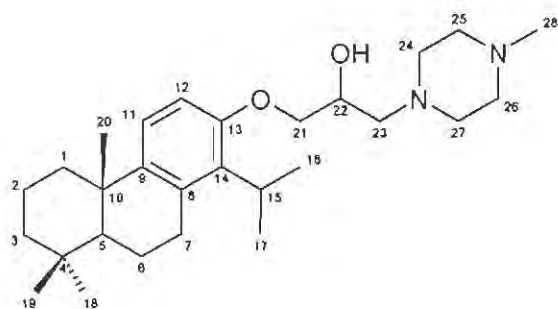
0.5g (3.47 mmol) of 2-naphthol (**19**), 0.125g (5.2 mmol) NaH and 1.35ml (17.3 mmol) of epichlorohydrin used, resulting oil purified via silica gel chromatography, eluting with Et₂O/Hex (3:7), *R_f* = 0.51, giving an isolated yield of 92% (632.6 mg). ¹H NMR δ_H (400 MHz; CDCl₃) 4.34 (dd, *J* = 11.0, 3.4, H-9a), 4.09 (dd, *J* = 10.8, 5.6, H-9b), 3.42 (m, H-10), 2.94 (dd, *J* = 4.8, 4.0, H-11α), 2.81 (dd, *J* = 5.2, 2.8, H-11β). ¹³C NMR δ_C (100.6 MHz; CDCl₃) 68.8 (C-9), 50.1 (C-10), 44.7 (C-11).

Synthesis of β-amino alcohols

General Procedure for synthesis of 5, 7, (8), 9, 12, 15-18, 21 and 22

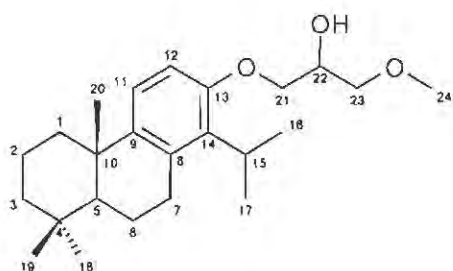
The respective amine (1.1 eq.) was added to 1 eq. of the respective epoxide in MeOH (1ml per 0.06 mmol starting material). The mixture was stirred at 51°C, for 12-24 hours, until the reaction was complete (verified via TLC). The excess solvent was removed under reduced pressure and the resulting oil purified using either preparative TLC or column chromatography.

Compound 5:



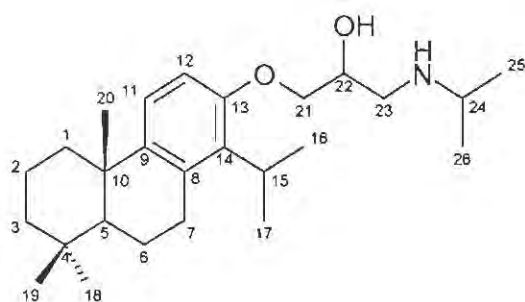
101.3mg (0.30 mmol) of totarol epoxide (**4**) used, 0.036ml (0.33 mmol) *N*-methylpiperazine used, resulting oil purified using preparative TLC, DCM/MeOH (9:1), $R_f = 0.56$, giving an isolated yield of 29% (38 mg). IR ν_{\max} (KBr)/ cm^{-1} 3134 (OH), 2805-2938 (CH aliphatic), 1590 (C=C aromatic); ^1H NMR δ_{H} (400 MHz; CDCl_3) 7.00 (d, $J = 8.8$, H-11), 6.51 (d, $J = 8.8$, H-12), 4.11 (m, H-22), 3.98 (dd, $J = 12.4$, 6.4, H-21a), 3.91 (dd, $J = 12.4$, 6.8, H-21b), 3.30 (sept, $J = 7.2$, H-15), 2.94 (dd, $J = 17.0$, 6.2, H-7 β), 2.75 (m, H-7 α), 2.57-2.60 (m, H-23), 2.34-2.56 (m, H-24, H-25, H-26, H-27), 2.30 (s, H-28), 2.23 (br d, $J = 11.6$, H-1 β), 1.92 (dd, $J = 7.7$, 1.6, H-6 α), 1.73 (dt, $J = 13.7$, 3.3, H-2 β), 1.55 – 1.70 (m, H-6 β), 1.49 (dt, $J = 13.2$, 3.2, H-2 α), 1.47 (br d, $J = 13.2$, H-3 β), 1.36 (d, $J = 7.1$, H-17), 1.34 (d, $J = 7.1$, H-16), 1.18 (s, H-20), 1.17-1.24 (m, H-1 α , H-3 α , H-5), 0.95 (s, H-18), 0.92 (s, H-19). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 155.1 (C-13), 143.3 (C-9), 133.7 (C-8), 133.2 (C-14), 122.7 (C-11), 109.8 (C-12), 80.0 (C-21), 70.1 (C-22), 65.9 (C-23), 65.7 (C-24), 61.0 (C-28), 55.1 (C-25), 53.2 (C-26), 49.5 (C-5), 45.9 (C-3), 41.5 (C-1), 39.6 (C-10), 37.6 (C-4), 33.1 (C-18), 28.7 (C-7), 27.4 (C-15), 22.9 (C-20), 21.5 (C-19), 20.6 (C-16, C-17), 19.4 (C-2), 19.3 (C-6), 17.0 (C-28); APCI m/z 443.5 (M^{+1}) $^+$. Found: C, 76.0; H, 10.0; N, 6.2. $\text{C}_{28}\text{H}_{46}\text{N}_2\text{O}_2$ requires C, 76.0; H, 10.5; N, 6.3. Mp 123°C.

Compound 8:



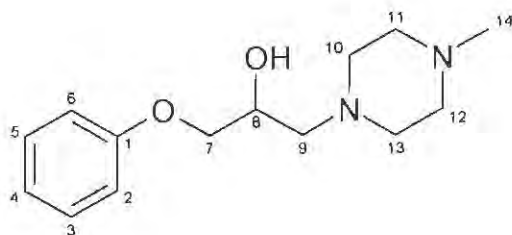
Resulted from prolonged exposure of the epoxide (4) with MeOH. IR ν_{\max} (KBr)/ cm^{-1} 3687 (OH), 2948 (C-H aliphatic), 1604, 1453 (C=C aromatic), 1117 (C-O); ^1H NMR δ_{H} (400 MHz; CDCl_3) 7.00 (d, $J = 8.8$, H-11), 6.51 (d, $J = 8.8$, H-12), 4.06-4.16 (m, H-22), 4.0 (dd, $J = 12.8$, 7.0, H-21a), 3.53-3.64 (m, H-23), 3.9 (dd, $J = 12.8$, 7.0, H-21b), 3.39 (s, H-24), 3.3 (sept, $J = 7.2$, H-15), 2.94 (dd, $J = 17.0$, 6.2, H-7 β), 2.75 (m, H-7 α), 2.23 (br d, $J = 12.1$, H-1 β), 1.92 (dd, $J = 7.7$, 1.6, H-6 α), 1.73 (dt, $J = 13.7$, 3.3, H-2 β), 1.55 – 1.70 (m, H-6 β), 1.49 (dt, $J = 13.2$, 3.2, H-2 α), 1.47 (br d, $J = 13.2$, H-3 β), 1.36 (d, $J = 7.1$, H-17), 1.34 (d, $J = 7.1$, H-16), 1.18 (s, H-20), 1.17-1.29 (m, H-1 α , H-3 α , H-5), 0.96 (s, H-18), 0.94 (s, H-19). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 143.5 (C-9), 133.1 (C-8), 122.8 (C-11), 109.9 (C-12), 73.8 (C-21), 68.7 (C-23), 69.2 (C-22), 59.2 (C-24), 49.5 (C-5), 41.5 (C-3), 39.6 (C-1), 37.6 (C-10), 33.2 (C-4), 33.1 (C-18), 29.6 (C-7), 28.7 (C-15), 25.1 (C-20), 21.5 (C-19), 20.7 (C-16, C-17), 19.4 (C-2), 19.3 (C-6), 14.1 (C-24); EIMS m/z 374 (M^+).

Compound 9:



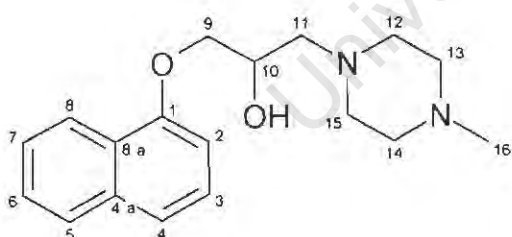
Reaction was performed at 31°C. 229.9mg (0.67 mmol) of totarol epoxide (**4**) used, 3 eq. (0.3ml, 3.52 mmol) isopropylamine used, resulting oil purified via silica gel chromatography, eluting with DCM/MeOH (9:1), $R_f = 0.37$, giving an isolated yield of 70% (189.3 mg). IR ν_{\max} (KBr)/cm⁻¹ 3313 (OH, H bonded), 2960, 2867 (C-H aliphatic), 1590 (C=C aromatic); ¹H NMR δ_H (400 MHz; CDCl₃) 7.09 (d, $J = 8.8$, H-11), 6.72 (d, $J = 8.8$, H-12), 4.11 (m, H-22), 3.96 (2 X ddd, $J = 9.4, 5.1, 1.8$, H-21a and b), 3.30 (br s, H-15), 2.84-3.00 (m, H-7 β), 2.76-3.00 (m, H-7 α , H-23), 2.74 (br s, H-24), 2.25 (d, $J = 12.4$, H-1 β), 1.92 (dd, $J = 13.3, 8.1$, H-6 α), 1.74 (dt, $J = 13.6, 3.3$, H-2 β), 1.63 – 1.72 (m, H-6 β), 1.59 (dt, $J = 13.6, 3.4$, H-2 α), 1.47 (br d, $J = 14.0$, H-3 β), 1.20-1.39 (m, H-1 α , H-3 α , H-5, H-16, H-17), 1.19 (s, H-20), 1.13 (d, $J = 6.4$, H-25, H-26), 0.95 (s, H-18), 0.93 (s, H-19). ¹³C NMR δ_C (100.6 MHz; CDCl₃) 155.1 (C-13), 143.5 (C-9), 133.8 (C-8), 133.2 (C-14), 122.8 (C-11), 110.0 (C-12), 70.2 (C-21), 69.6 (C-22), 49.61 (C-23), 49.59 (C-24), 49.5 (C-5), 49.1 (C-24), 41.6 (C-3), 39.6 (C-1), 37.7 (C-10), 33.2 (C-4), 33.2 (C-18), 28.7 (C-7), 27.4 (C-15), 25.1 (C-16), 22.7 (C-17), 21.6 (C-25), 20.7 (C-26), 20.7 (C-20, C-19), 19.5 (C-2), 19.4 (C-6); APCI m/z 402.5 (M^{+1})⁺. Found: C, 77.3; H, 10.2; N, 3.2. C₂₆H₄₃NO₂ requires C, 77.8; H, 10.8; N, 3.5. Mp 39-42°C.

Compound 12:



500mg (3.61 mmol) of phenyl epoxide (**11**) used, 0.44ml (3.97 mmol) *N*-methylpiperazine used, resulting oil purified via silica gel chromatography, eluting with DCM/MeOH (9:1), $R_f = 0.43$, giving an isolated yield of 67% (608.3 mg). IR ν_{\max} (KBr)/ cm^{-1} 3348 (OH H-bonded), 2938, 2806 (C-H aliphatic), 1596 (C=C aromatic). ^1H NMR δ_{H} (400 MHz; CDCl_3) 4.05-4.13 (m, H-8), 3.98 (d, $J = 5.1$, H-7), 2.73 (br d, $J = 5.4$, H-9), 2.59-2.51 (m, H-10, H-11, H-12, H-13), 2.31 (s, H-14). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 70.2 (C-8), 65.6 (C-7), 60.5 (C-9), 55.1 (C-10, C-13), 53.1 (C-11, C-12), 45.9 (C-14); APCI m/z 251.2 (M^{+1}) $^+$. Found: C, 66.2; H, 8.6; N, 11.0. $\text{C}_{14}\text{H}_{22}\text{N}_2\text{O}_2 \cdot 0.2\text{H}_2\text{O}$ requires C, 66.2; H, 8.7; N, 11.0. Mp 51-60°C.

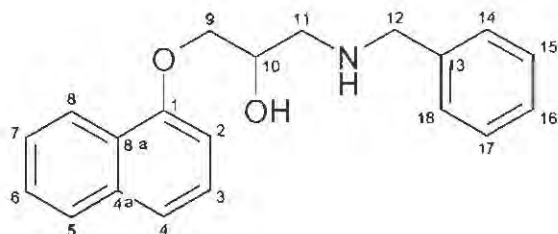
Compound 15:



190mg (0.95 mmol) of 1-naphthyl epoxide (**14**) used, 0.12ml (1.1 mmol) *N*-methylpiperazine used, resulting oil purified via silica gel chromatography, eluting with MeOH, $R_f = 0.26$. The compound was further purified via recrystallization with hexane and dried under high vacuum giving an isolated yield of 68% (192.3 mg). IR ν_{\max} (KBr)/ cm^{-1} 3162 (OH), 3058 (C-H aromatic), 2964-2923, 2878-2782 (C-H aliphatic), 1578 (C=C aromatic). ^1H NMR δ_{H} (400 MHz; CDCl_3) 4.19-4.25 (m, H-9a and b), 4.14-4.16 (m, H-10),

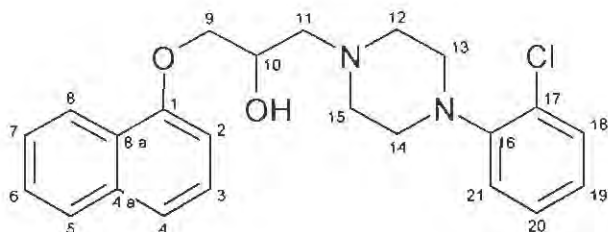
2.67-2.69 (m, H-11a and b), 2.76 (br s, H-12, H-15), 2.50 (br s, H-13, H-14), 2.31 (s, H-16). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 70.6 (C-9), 65.7 (C-10), 60.8 (C-11), 55.2 (C-12, C-15), 53.3 (C-13, C-14), 46.0 (C-16). APCI m/z 301.1 (M^+) $^+$. Found: C, 70.2; H, 7.7; N, 9.0. $\text{C}_{18}\text{H}_{24}\text{N}_2\text{O}_2 \cdot 0.5\text{H}_2\text{O}$ requires C, 69.9; H, 7.8; N, 9.1. Mp 66-77°C.

Compound **16**:



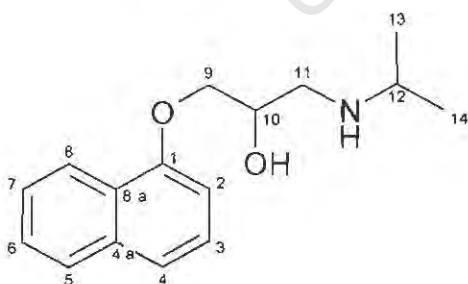
0.89 mmol of 1-naphthyl epoxide (**14**) used, 1.1 mmol benzylamine used, resulting oil purified via silica gel chromatography, eluting with EtOAc/Hex (1:1), R_f = 0.63, giving an isolated yield of 34% (93.4 mg). IR ν_{max} (KBr)/ cm^{-1} 3279 (OH H-bonded), 3050 (C-H aromatic), 2942 (C-H aliphatic), 1580 (C=C aromatic). ^1H NMR δ_{H} (400 MHz; CDCl_3) 7.26-7.51 (m, H-3, H-4, H-6, H-7, H-14 to 18), 4.29 (m, H-10), 4.10-4.21 (m, H-9a and b), 3.93 (d, J = 4, H-12), 2.92-3.06 (m, H-11a and b), 2.88 (NH). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 138.4 (C-13), 128.6 (C-15, C-17), 128.5 (C-14, C-18), 127.5 (C-16), 70.5 (C-10), 68.1 (C-9), 53.5 (C-12), 51.2 (C-11). APCI m/z 308.4 (M^+) $^+$. Found: C, 78.1; H, 6.6; N, 4.6. $\text{C}_{20}\text{H}_{21}\text{NO}_2$ requires C, 78.2; H, 6.9; N, 4.6. Mp 112-113°C.

Compound 17:



Neutralization of 1-(2-chlorophenyl)-piperazine monohydrochloride (271mg, 2.40 mmol) with MP-carbonate (3.65mg, 9.6 mmol) in MeOH (7ml) at 20°C for 7 hours yielded amine. 134.4mg (0.67 mmol) of 1-naphthyl epoxide (**14**) used. Resulting oil purified via silica gel chromatography, eluting with EtOAc/Hex (1:1), $R_f = 0.72$, giving an isolated yield of 55% (147 mg). IR ν_{\max} (KBr)/ cm^{-1} 3345 (OH H-bonded), 3054 (C-H aromatic), 2807-2933 (C-H aliphatic), 1579 (C=C aromatic). ^1H NMR δ_{H} (400 MHz; CDCl_3) 7.26 (d, $J = 0.9$, H-18), 7.24 (m, H-20), 7.21 (dd, $J = 8.1, 1.8$, H-21), 7.02 (td, $J = 7.6, 1.6$, H-19), 4.35 (quintet, $J = 8.6$, H-10), 4.15-4.28 (m, H-9a and b), 3.16 (m, H-12, H-15), 2.752-2.819 (m, H-13, H-14), 2.94-3.00 (m, H-11a and b). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 149.0 (C-16), 130.7 (C-18), 128.8 (C-17), 125.6 (C-20), 123.9 (C-21), 121.9 (C-19), 70.5 (C-10), 65.6 (C-9), 61.1 (C-12, C-15), 53.6 (C-13, C-14), 51.1 (C-11). APCI m/z 397.3 (M^+) $^+$. HPLC indicated that the compound was pure. Mp 107-117°C.

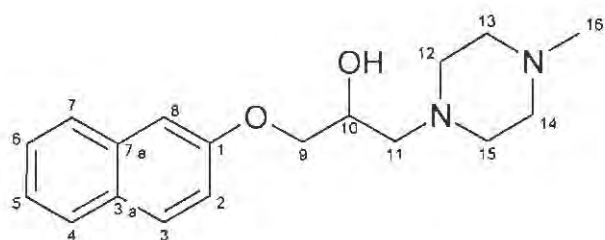
Compound 18:



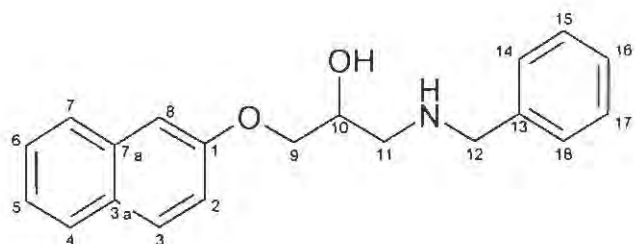
Reaction was performed at 31°C. 167mg (0.83 mmol) of 1-naphthyl epoxide (**14**) used, 3 eq. (0.30ml, 3.52 mmol) isopropylamine used, resulting oil purified via silica gel chromatography, eluting with EtOAc, $R_f = 0.11$, giving an isolated yield of 93% (200.6 mg).

IR ν_{\max} (KBr)/ cm^{-1} 3271 (OH, H-bonded), 3054 (C-H aromatic), 2963, 2923, 2834 (C-H aliphatic), 1583 (C=C aromatic). ^1H NMR δ_{H} (400 MHz; CDCl_3) 4.13-4.21 (m, H-10, OH), 3.90 (m, H-9a), 3.62 (dd, $J = 11.2, 3.2$, H-9b), 3.39 (dd, $J = 11.0, 7.8$, H-11a), 3.01 (dd, $J = 16, 4.8$, H-11b), 2.88 (m, H-12), 2.20 (NH), 1.11 (d, $J = 3.1$, H-13, H-14). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 70.7 (C-10), 68.5 (C-9), 68.0 (C-11), 49.0 (C-12), 23.0 (C-13), 22.9 (C-14). APCI m/z 260.3 (M^{+1}) $^+$. Found: C, 71.7; H, 7.9; N, 4.9. $\text{C}_{16}\text{H}_{21}\text{NO}_2 \cdot 0.5\text{H}_2\text{O}$ requires C, 71.6; H, 7.9; N, 5.2. Mp 35-79°C.

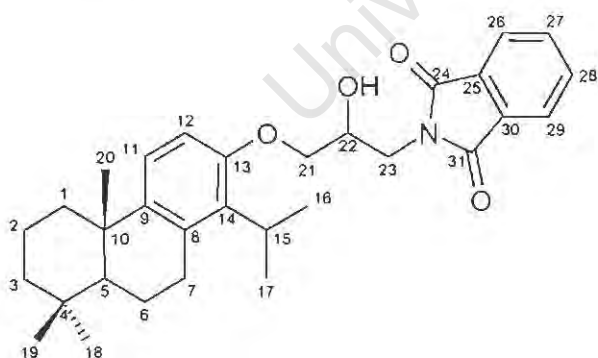
Compound 21:



313.7mg (1.57 mmol) of 2-naphthol epoxide (**20**) used, 0.19ml (1.72 mmol) *N*-methylpiperazine used, resulting oil purified via silica gel chromatography, eluting with MeOH, $R_f = 0.18$. The compound was further purified via recrystallization with hexane and EtOAc and dried under high vacuum giving an isolated yield of 48% (226.2 mg). IR ν_{\max} (KBr)/ cm^{-1} 3365 (OH), 3025 (C-H aromatic), 2942, 2807 (C-H aliphatic), 1631-1600 (C=C aromatic). ^1H NMR δ_{H} (400 MHz; CDCl_3) 4.16 (m, H-10), 4.10 (d, $J = 3$, H-9a and b), 2.73 (br d, $J = 5.7$, H-12, H-15), 2.63-2.59 (m, H-11a and b), 2.50 (s, H-13, H-14), 2.31 (s, H-16). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 70.4 (C-10), 65.6 (C-9), 60.5 (C-12, C-15), 55.1 (C-11, C-15), 53.1 (C-13, C-14), 45.9 (C-16). APCI m/z 301.3 (M^{+1}) $^+$. Found: C, 68.6; H, 7.2; N, 9.1. $\text{C}_{18}\text{H}_{24}\text{N}_2\text{O}_2 \cdot 0.8\text{H}_2\text{O}$ requires C, 68.9; H, 7.7; N, 9.0. Mp 132°C.

Compound **22**:

225.7mg (1.13 mmol) of 2-naphthol epoxide (**20**) used, 0.135ml (1.24 mmol) benzylamine used, resulting oil purified via silica gel chromatography, eluting with MeOH, R_f = 0.47, giving an isolated yield of 67% (230.7 mg). IR ν_{\max} (KBr)/ cm^{-1} 3266 (OH H-bonded), 3053 (C-H aromatic), 2716-2899 (C-H aliphatic), 1601-1629 (C=C aromatic). ^1H NMR δ_{H} (400 MHz; CDCl_3) 7.44 (dt, J = 10.2, 1.6, H-14, H-18) 7.13-7.36 (m, H-2, H-5, H-6, H-8, H-15, H-16, H-17), 4.19 (m, H-10), 4.10 (d, J = 6.4, H-9a and b), 3.89 (br s, H-12a and b), 2.84-3.00 (m, H-11a, b and NH). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 128.5 (C-14, C-18), 128.3 (C-15, C-17), 127.3 (C-16), 70.4 (C-10), 68.2 (C-9), 53.6 (C-12) 51.1 (C-11). APCI m/z 308.4 (M^+) $^+$. Found: C, 77.3; H, 6.6; N, 4.5. $\text{C}_{20}\text{H}_{21}\text{NO}_2 \cdot 0.1\text{H}_2\text{O}$ requires C, 77.7; H, 6.8; N, 4.5. Mp 95-102°C.

Compound **6**:

Compound **4** (294.3 mg, 0.86 mmol), potassium phthalimide (7.7 mg, 0.04 mmol) and phthalimide (142.2 mg, 0.946 mmol) were dissolved in DMF (13 ml). The reaction mixture was stirred for 12 hours at 90°C. Completion of reaction was confirmed by TLC, $\text{Et}_2\text{O}/\text{Hex}$

(3:10), $R_f = 0.15$. The reaction mixture was diluted with water (50 ml) and extracted with CHCl_3 (3 X 50 ml). The extracts were washed with water (2 X 50 ml) and brine (2 X 50 ml) and dried over MgSO_4 . The solvent was removed under reduced pressure to give a yellowish oil. The oil was dissolved in EtOAc (100 ml), washed with water (3 X 100 ml), dried and loaded onto a silica gel column. The column was eluted with $\text{Et}_2\text{O}/\text{Hex}$ (3:10) and once the spot of interest had been eluted, baseline material was washed out with $\text{Et}_2\text{O}/\text{Hex}$ (1:1). Fractions were concentrated under reduced pressure and dried on high vacuum, resulting in 0.25g (63%) of compound **6**. IR ν_{max} (KBr)/ cm^{-1} 3417 (OH), 2930 (CH aliphatic), 1702 (C=O), 1443 (C=C aromatic), 1263 (C-O), 1073 (C-N); ^1H NMR δ_{H} (400 MHz; CDCl_3) 7.88 (dd, $J = 5.6, 3.0$, H-26, H-29), 7.73 (dd, $J = 5.4, 3.2$, H-27, H-28) 7.00 (d, $J = 8.9$, H-11), 6.51 (d, $J = 8.9$, H-12), 4.32 (m, H-22), 4.03 (m, H-21, H-23), 3.34 (sept, $J = 7.2$, H-15), 2.94 (dd, $J = 17.2, 6.2$, H-7 β), 2.75 (m, H-7 α), 2.25 (br d, $J = 11.2$, H-1 β), 1.92 (dd, $J = 7.7, 1.6$, H-6 α), 1.73 (dt, $J = 17.0, 3.4$, H-2 β), 1.55 – 1.70 (m, H-6 β), 1.47 (dt, $J = 13.2, 3.2$, H-2 α), 1.47 (br d, $J = 13.2$, H-3 β), 1.35 (dd, $J = 10.1, 3.6$, H-16, H-17), 1.18 (s, H-20), 1.18-1.26 (m, H-1 α , , H-3 α , H-5), 0.95 (s, H-18), 0.92 (s, H-19). ^{13}C NMR δ_{C} (100.6 MHz; CDCl_3) 168.7 (C-24, C-31), 154.9 (C-25, C-30), 143.8 (C-13), 134.1 (C-26, C-29), 133.4 (C-27, C-28), 132.0 (C-14), 123.5 (C-11), 122.9 (C-12), 109.9 (C-22), 69.6 (C-21), 69.3 (C-5), 49.5 (C-3), 41.6 (C-23), 41.5 (C-1), 39.6 (C-10), 37.7 (C-4), 33.3 (C-18), 33.2 (C-7), 29.7 (C-15), 28.8 (C-16), 25.2 (C-17), 21.6 (C-20), 20.9 (C-19), 19.5 (C-2), 19.4 (C-6); m/z 512 ($\text{M}^{+\text{Na}}$) $^+$. HPLC indicated that the compound was pure. Mp 148°C.

4.2. Antiplasmodial activity and Cytotoxicity testing

4.2.1. Determination of *in vitro* antiplasmodial activity

4.2.1.1. Culturing of *Plasmodium falciparum* parasites

Strains were cultured according to methods described by Trager and Jensen (1976). The chloroquine sensitive clone, D10 (originated from FCQ-27 from Papua New Guinea) [Ekong *et al.*, 1993], and chloroquine resistant strain, K1 (Kanchanaburi, Thailand) [Thaitong *et al.*, 1981], were maintained at a 4% hematocrit in RPMI 1640 (Biowhittaker) medium supplemented with Albumax II (GIBCO/Invitrogen), HEPES, gentamicin and sodium bicarbonate (Sigma). The medium was changed daily. The parasitaemia was kept below 10% with erythrocytes obtained from O⁺ blood (Western Province Blood Transfusion Service, Groote Schuur Hospital, Cape Town, South Africa). Medium without Albumax II was used to wash the blood in order to obtain packed erythrocytes. The parasites were synchronized when in the ring stage with sorbitol, as described by Lambros and Vanderberg (1979). The cultures were maintained in flat blotted 75 cm³ flasks in an atmosphere of 93% N₂, 4% CO₂ and 3% O₂ at 37°C.

4.2.1.2. Experiment and plate setup

Chloroquine control: A 10mg/ml stock solution of CQ (Sigma) was made up and serially diluted to 2 µg/ml with medium, which was loaded onto the plate.

Compounds: The compounds were dissolved in either methanol (MeOH) or dimethylsulfoxide (DMSO) (Table 4.2.1.2.1.) and made up to a 1mg/ml stock solution with Millipore water. The solvent and the ratio in which it was used was chosen according to each compound's solubility. Controls were prepared using the various solvents at the same concentrations used in the assay to ensure that the solvent did not influence the observed IC₅₀.

Table 4.2.1.2.1.: The various solvent ratios in which compounds **3-22** were dissolved.

Compound	Solvent	Ratio of solvent to water
3	MeOH	1:4
4	MeOH	1:4
5	MeOH	1:9
6	DMSO	1:4
7	DMSO	1:4
8	DMSO	1:4
9	MeOH	1:4
10	MeOH	1:4
11	MeOH	1:4
12	MeOH	1:9
13	MeOH	1:4
14	DMSO	1:4
15	MeOH	1:9
16	MeOH	1:4
17	MeOH	1:4
18	MeOH	1:4
19	MeOH	1:4
20	DMSO	1:4
21	MeOH	1:9
22	MeOH	1:4

The stock solutions were sonicated thoroughly before water was added to ensure the compounds had fully dissolved and that the solutions were sterile. It was found that if the solutions were sonicated after addition of water, the compounds were more likely to

precipitate. All solutions were stored at 20°C, and new solutions were made up every couple of weeks.

All compounds were initially tested for *in vitro* antiplasmodial activity on the chloroquine sensitive (D10) strain of *P. falciparum*. Compounds showing activity greater than that of totarol (compounds **5–9**, **16**, **22**) were then tested on a chloroquine resistant (K1) strain. Compounds **12**, **15**, **18** and **21** were tested for comparative purposes. The selectivity of compounds showing activity greater than that of totarol in the K1 strain (compounds **5**, **7–9**, **15**, **16**, **18**, **21** and **22**) were tested for cytotoxicity. Compound **12** was tested for comparative purposes.

The experiment was performed in 96 well microtitre plates. These plates contain 96 wells, which are arranged in 12 columns (1–12) and 8 rows (A–H). 200µl of the chloroquine control was loaded into the first two wells of column 3 (i.e. 3A and 3B). 200µl of the respective compounds, diluted to the appropriate concentration in medium, were loaded in duplicate in the remaining wells of column 3. 100µl of medium was added to all wells except those in column 3. Using a multichannel pipette, 100µl of the drug solution was removed from every well in column 3 and added to column 4 and mixed. These series of dilutions continue until the end of the plate. 100 µl of parasitised red blood cells (2% hematocrit and 2% parasitaemia) was then added to each well in columns 2–12. This halves the concentration of the compound; the highest concentration of compound to which the parasites were exposed is 100µg/ml and the highest concentration of chloroquine was 1µg/ml. 100µl of non-parasitised red blood cells (2% hematocrit) are added to the wells in column 1 (blank). The total volume in each well is 200µl. The plate is then gassed and incubated at 37°C for 48 hours. Column 1 serves as a blank and contains only red blood cells at a final hematocrit of 1%. Column 2 contains no drugs, while columns 3 to 12 contain parasites and a range of drug concentrations. The experiment is carried out on separate occasions in duplicate until three reproducible results are obtained.

4.2.1.3. Parasite lactate dehydrogenase assay

The lactate dehydrogenase assay differentiates between live and dead parasites by measuring the activity of the parasite lactate dehydrogenase enzyme (pLDH). pLDH activity is differentiable from the erythrocyte lactate dehydrogenase (LDH) activity as it uses an analog of nicotinamide adenine dinucleotide (NAD^+), called 3-acetyl pyridine adenine dinucleotide (APAD $^+$) much faster than LDH does. This results in the production of APADH which reduces nitroblue tetrazolium producing formazan crystals from which the amount of live parasites is then determined spectrophotometrically [Makler *et al.*, 1993].

100 μl of Malstat reagent (triton X-100, 1mg/ml, APAD, 0.33g/L, TRIS buffer, 3.3g/L in millipore water and lactate) is transferred to all wells in a new microtitre plate. All wells in the incubated plate are resuspended, and 15 μl of the various 'solutions' from each column are then transferred into the plate containing 100 μl of Malstat reagent. 25 μl of a solution containing nitroblue tetrazolium, 1.96 mM (Sigma) and phenazine ethosulfate, 0.24 mM (Sigma) in millipore water was added and the absorbance of the formazan salt is read by a 7520 Microplate Reader (Cambridge Technology) at 620nm.

4.2.1.4. Data analysis

The % parasite viability was calculated using the formula below:

$$\% \text{ parasite viability} = \frac{\text{Absorbance test well (columns 3-12)}}{\text{Absorbance of the positive control (column 2)}} \times 100$$

The results were analyzed using Microsoft Excel 2000 and Prism 4.0.

The resistance index [$R_i = \text{IC}_{50} (\text{K1}) / \text{IC}_{50} (\text{D10})$] for compounds tested against both D10 and K1 strains was determined. The R_i gives an indication of the difference in activity of the various compounds in a sensitive and resistant strain of *P. falciparum* and is used as a factor to evaluate whether novel antimalarials have potential activity against CQ resistant parasites.

The dose response curves are in appendix 2.

4.2.2. Determination of cytotoxicity

4.2.2.1. Chinese Hamster Ovarian cell culture

Chinese ovarian cells were maintained in culture and medium was changed daily. The medium consisted of Dulbecco's Modified Eagles' Medium (DMEM): Hams (1:1) (Sigma) supplemented with heat inactivated fetal calf serum (FCS) (10%) and gentamicin (0.04 µg/ml), which were all purchased from Highveld Biological, South Africa. The cells were maintained in 75cm³ flasks in a 5% CO₂ humidified atmosphere at 37°C. Subculturing was performed once the cells were confluent. A 0.25% trypsin solution was used for subculturing and cells only underwent a maximum of 10 subcultures.

4.2.2.2. Experiment and plate setup

Emetine control: The 2mg/ml stock solution was diluted in medium until a concentration of 2ng/ml was obtained.

Compounds: Only those compounds showing improved activity when compared to totarol against both the D10 and K1 strains of *P. falciparum* were tested. The 1mg/ml stock solutions were diluted with complete medium to a concentration of 200 µg/ml. This was then further diluted in a series of 10 fold dilutions to a lowest concentration of 0.002 µg/ml. Experiments were performed using a cell concentration of 10⁵ cells/ml.

Culture plates (96 well) from Costar were used for the experiment. 100µl of cell suspension (mix between each row) and 100µl of medium are added to columns A-G. 200µl of CM is added to column H (blank). Column G is the positive control. Columns A-F will contain the compounds. The plate is then incubated overnight (24 hours). After this time period, the plate is checked under the microscope to see if the cells have settled and are in the exponential phase. The medium is then removed from the wells via aspiration and 100µl of the various concentrations of emetine or compounds is loaded in triplicate. 100µl of medium is then added to each well so the emetine or compound concentration in each well is halved. The highest drug concentration to which the cells were exposed was

100 µg/ml (column F) and the lowest concentration was 0.001 µg/ml (column A). The plate is then incubated for 48 hours.

The experiment was performed on separate occasions in triplicate and repeated until two reproducible results were obtained.

4.2.2.3. MTT assay

The MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay measures the amount of viable cells indirectly by using the metabolic activity of mitochondria which are only present in viable cells. On addition of a yellow water-soluble tetrazolium salt, the mitochondria (within viable cells) metabolize this yellow salt into a purple water-insoluble formazan product. The amount of purple formazan product produced is proportional to the number of active mitochondria and therefore the number of viable cells [Mosmann, 1983].

After the 48 hour incubation period, 25µl of MTT (Sigma, 5mg/ml in Millipore water) was added to each well. The plate is then incubated for a further 4 hours. On removal of the plate, purple crystals will be observed. These are the formazan product and indicate that viable cells are present. The plate is then spun at 200 rpm for 10 minutes, after which the medium is carefully aspirated off. 100µl of DMSO (dissolves formazan crystals, so the absorbency can be read) is added and the plates are shaken for 3-5 minutes to assist in the solvation of the crystals. The plates are then read on a Microtitre Plate Reader at 540nm.

4.2.2.4. Data analysis

The % cell viability is calculated as shown below;

$$\% \text{ Cell viability} = \frac{\text{Absorbance test well (columns F-A)}}{\text{Absorbance of positive control (column G)}} \times 100$$

The results were analyzed using Microsoft Excel 2000 and Prism 4.0.

The selectivity index [$S_I = IC_{50} \text{ (CHO)} / IC_{50} \text{ (D10/K1)}$], gives an indication as to how selective compounds are towards *P. falciparum*, and it was determined for compounds tested for cytotoxicity.

The dose response curves are shown in appendix 2.

4.3. Effect of compounds 5 and 7 on tritiated chloroquine accumulation

This experiment is carried out in triplicate. Parasites (in trophozoite stage with a parasitemia of 5% and a hematocrit of 1%) in 1.5ml microcentrifuge tubes are incubated for 15 minutes at 37°C in the presence of various concentrations of compounds **5**, **7** and verapamil (positive control; Sigma) after which [^3H]CQ (Moravek, USA) was added (final concentration 4nM). The solutions are then heated for an hour, shaking them after half an hour. 100 μl of dibutylphthalate (DBP) (Sigma) is then added to separate the parasitised red blood cells and the unparasitised red blood cells from the rest of the mixture. The mixtures are spun for 15 seconds and the supernatant is aspirated off. The microcentrifuge tip containing the parasites is cut off and placed in a scintillation vial. 100 μl of Solvable (PerkinElmer) is added which digests the pellet. 25 μl of EDTA (0.1M) is added to disable catalase to prevent it from interfering with the peroxide which is used to bleach the mixture. 100 μl of peroxide (30%), followed by 2mls of scintillation fluid (Packard Bioscience), is added to each tube. The radioactivity within the various mixtures was counted using a Packard Tri-Carb 2100TR liquid scintillation spectrophotometer. The experiment is performed on separate occasions until two reproducible results were obtained.

4.4. Chloroquine potentiating effect of 5 and 7

The chloroquine potentiating ability of each compound should be determined at a concentration which has optimum chloroquine uptake and is non-toxic to the parasite. The chloroquine-resistant K1 strain of *P. falciparum* is used. Plates are setup containing CQ, as described in section 4.2.1.2. A calculated amount of the prospective chemosensitizers are added to each well, as well as a verapamil control (1 μM). The plate is incubated for 48 hours [Adaptation of Chawira and Warhurst, 1987]. The IC_{50} of the parasites in the presence of the prospective chemosensitizers was measured using the parasite lactate

dehydrogenase (pLDH) assay [Makler *et al.*, 1993] as described in section 4.2.1.3.. The experiment is performed in duplicate on separate occasions until three reproducible results were obtained.

4.5. Measurement of erythrocytic lysis

This is determined indirectly by quantifying the amount of haemoglobin (released when cells lyse) in the medium. Dose response experiments of selected compounds were performed as described in 4.2.1.2. although only RBC at a hematocrit of 2% were used. The plates were incubated for 48 hours, after which the erythrocytes were spun down and the supernatant was removed and transferred to a separate 96 well plate. The amount of haemoglobin was measured on a Microtitre Plate Reader at 405nm.

4.6. Erythrocytic morphology effect assay

Dose response experiments of selected compounds were performed as described in 4.2.1.2. although only RBC, at a hematocrit of 2% were used. After a 48 hour incubation period, the plate was placed on a shaker for approximately 2 minutes. The erythrocytes were fixed by adding 100 μ l of 1% gluteraldehyde (Sigma) in 3% paraformaldehyde to 100 μ l of erythrocyte suspension from each well. The erythrocytes were examined and photographed by phase contrast light microscopy at X1000.

4.7. Invasion assay

Parasites used were in the schizont stage of development with a hematocrit of 1% and parasitemia of 2%. Parasites were exposed to the selected compounds at their respective IC₅₀ values until all obvious schizonts had burst and a new ring stage was evident in the control culture. Parasite viability was determined via Giemsa stained smears. A minimum number of 1000 cells were counted [Haldar *et al.*, 2003]. The experiment was performed on three separate occasions.

Chapter 5

***Effect of Incorporation of a β -Amino Group
on In Vitro Antiplasmodial Activity and
Cytotoxicity of Totarol***

5.1. Introduction

The effect of the incorporation of β -amino groups with different substitutions and side chains will be examined in this section. The β -amino alcohol portion of the molecule is a potent pharmacophore present in quinine and mefloquine (Figure 1.6.2.2.1.), and it is therefore likely that it plays an important role in the observed antiplasmodial activities of the synthesized derivatives.

5.2. Results

The effect of the β -amino group on the antiplasmodial activity of tatarol was investigated and the results are shown below in Table 5.2.1.. Compounds synthesized for this study are shown in Figure 5.2.1..

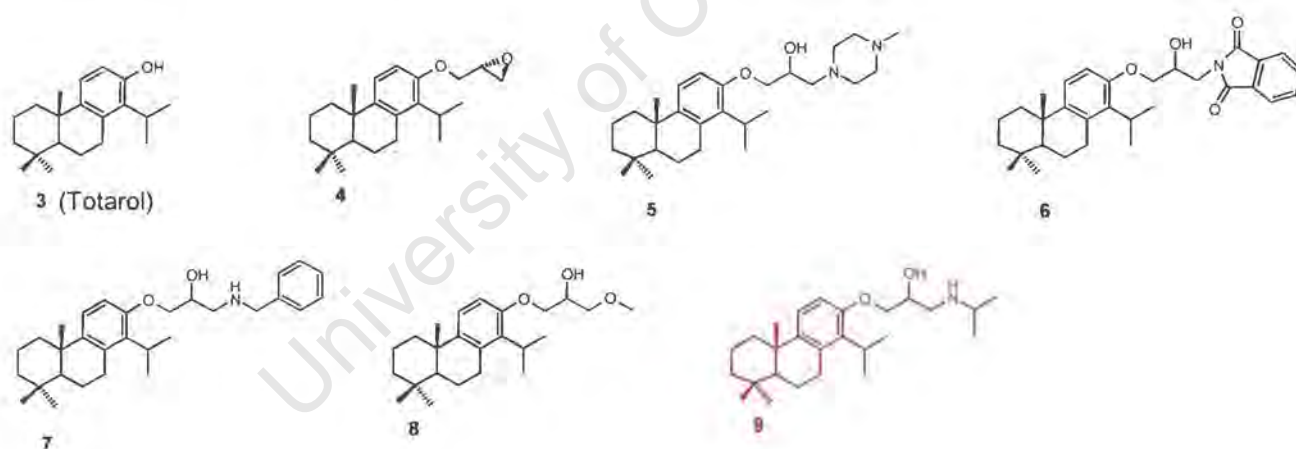
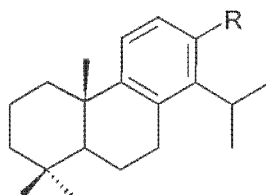


Figure 5.2.1.: Structure summary of tatarol (3) and synthesized derivatives 4-9.

(Note: Although compound 9 was synthesized for investigation into its effect on erythrocyte invasion, it will be discussed with reference to its β -amino alcohol side chain).

Table 5.2.1.: Summary of IC_{50} 's of all compounds as well as the calculated R_i and S_i values [$R_i = IC_{50} (K1)/IC_{50} (D10)$; $S_i = IC_{50} (CHO)/IC_{50} (K1)$].



Compound	Side-chain (R)	IC_{50} $\mu g/ml$ (μM)			R_i	S_i	
		D10	K1	CHO		D10	K1
3 (Totarol)	-	3.38 ± 0.72 (11.78 ± 2.53)	3.35 ± 0.22 (11.69 ± 0.78)	48.79 ± 0.96 (170.46 ± 3.35) [Clarkson <i>et al.</i> , 2003b]	0.99	14.5	14.6
4		3.54 ± 0.57 (10.33 ± 1.66)	4.81 ± 1.64 (14.04 ± 4.80)	ND	1.36	-	-
5		0.60 ± 0.10 (1.36 ± 0.24)	0.62 ± 0.12 (1.39 ± 0.26)	1.65 ± 0.09 (3.73 ± 0.20)	1.03	2.7	2.7
6		4.68 ± 0.33 (9.55 ± 0.68)	23.66 ± 9.29 (48.33 ± 18.98)	ND	5.06	-	-
7		2.91 ± 0.89 (6.47 ± 1.99)	0.29 ± 0.12 (0.65 ± 0.27)	6.48 ± 0.22 (14.41 ± 0.49)	0.1	2.2	22.3
8		2.97 ± 0.13 (7.92 ± 0.35)	3.52 ± 0.22 (9.41 ± 0.60)	34.86 ± 3.96 (93.07 ± 10.57)	1.19	11.8	9.9
9		0.42 ± 0.09 (1.05 ± 0.22)	0.06 ± 0.01 (0.17 ± 0.03)	0.52 ± 0.21 (1.28 ± 0.51)	0.14	1.2	8.7

ND = Not determined (Activity was not greater than that of totarol)

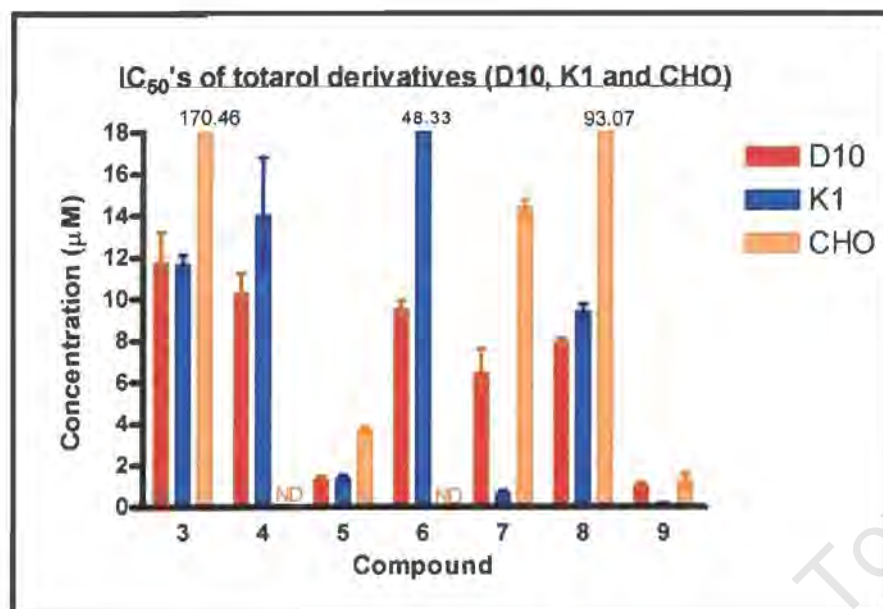


Figure 5.2.2.: Summary of totarol (3) and derivatives (4-9) IC₅₀ values in D10, K1 and CHO (ND = Not determined as activity was not greater than that of totarol).

In general all derivatives synthesized have activity better than that of totarol in both CQ sensitive (D10) and CQ resistant (K1) strains. The derivatives' respective activities are discussed below.

5.3. Discussion

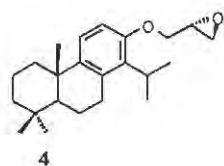
Totarol (3)

A number of (+)-totarol's antibacterial actions are thought to be mediated by its interaction with the cell membrane. It has been shown that (+)-totarol incorporates into membranes very efficiently, as would be expected due to its hydrophobic character [Mateo *et al.*, 2000; Micol *et al.*, 2001], thereby disturbing membrane structure. (+)-Totarol has been shown to orientate itself in the phospholipid bilayer with its phenol group facing inwards (i.e. into the membrane), thereby disrupting phospholipid packing and interaction [Mateo *et al.* 2000]. Disruption of the membrane may also be a possible mode of action for (+)-totarol and its derivatives in *P. falciparum*, especially with compounds whose IC₅₀ values are similar in

chloroquine sensitive and resistant strains (compounds **4**, **5** and **8**). Totarol and its derivatives are also lipophilic, as indicated by their clog P values (appendix 2) which are all greater than 5, and are therefore likely to target the cell membrane.

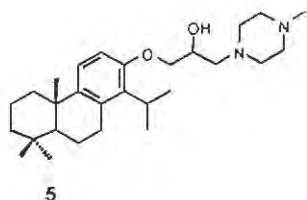
Cytotoxicity experiments indicate that totarol is highly selective [Clarkson *et al.*, 2003b]. This leads to the suggestion that totarol, if it is orientated in the membrane, perhaps binds to a target (e.g. membranous protein) found in *P. falciparum* and not in Chinese Hamster Ovarian cells.

Compound 4



The activity of compound **4** is very similar to that of totarol, although its resistance index is slightly higher. It is therefore likely that the mechanism of action of these two compounds is similar. The increased activity of compounds **5**, **7**, **8** and **9** in both strains and **6** in the D10 strain relative to the activity of compound **4**, can therefore be attributed to the compounds' respective side chains, which perhaps increase binding to their target molecule(s).

Compound 5

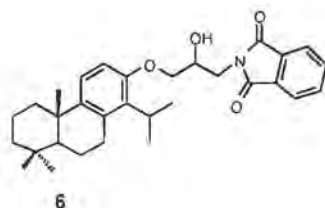


Compound **5** was shown to have one of the highest activities out of all the derivatives synthesized in both D10 and K1 strains. It is approximately 8 times more effective than totarol in both these strains. The structure of compound **5** was designed to contain two protonatable nitrogens which would possibly allow the compound to accumulate within the

acidic food vacuole due to the weak base effect (as seen with chloroquine). Compound **5** displayed similarly high activity in both sensitive and resistant strains as indicated by the resistant index ($R_i = 1.03$). The similarity in IC_{50} values between the chloroquine sensitive and resistant strains indicates that the mechanisms by which chloroquine resistance is conferred do not affect the accumulation or activity of **5** in the parasite.

The selectivity index of **5** is very low, indicating that it is a toxic compound with relatively low specific antiplasmodial activity. This toxicity may be caused by the basic terminal NMe moiety since related compounds with a less basic N-Aryl moieties are significantly less cytotoxic [Clarkson *et al.*, 2003b].

Compound 6

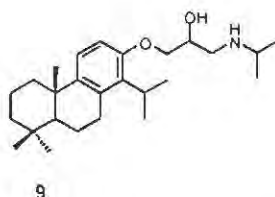
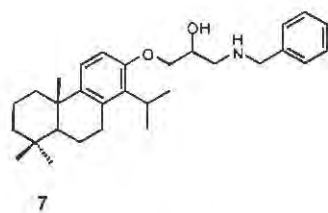


Compound **6** is an imide but may also be regarded as a tertiary β -amino alcohol derivative. It has the highest IC_{50} for both strains. The tertiary nitrogen in this compound is unlikely to become protonated as its electron pair is not available due to delocalisation of the electrons into the adjacent carbonyl groups. Comparison with the other tertiary β -amino alcohols, such as **5-9**, leads to the suggestion that a protonatable tertiary nitrogen is necessary for activity. Compound **6** also has a R_i of 5.06, indicating that this compound is more active in the sensitive strain. This may be due to the compound;

- i) Not reaching its target in the resistant strain.
- ii) The target may be altered in the resistant strain.

The structures of chloroquine and **6** differ considerably and hence it would be unlikely that resistance to **6** was due to cross resistance with chloroquine.

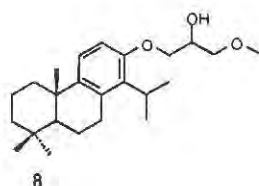
Compounds 7 and 9



Compounds **7** and **9** are secondary β -amino alcohols. They have a R_1 of 0.1 and are therefore much more active in the K1 strain than in the D10 strain. Mefloquine, halofantrine and quinine also display a similar pattern of activity as these two compounds in D10 and K1 strains, which is opposite to that seen with CQ [Cowman *et al.*, 1994]. Mefloquine resistance has been correlated with resistance to other amino-alcohol containing antimalarials and linked to an amplification and upregulation of *pfmdr1*. This suggests that the D10 resistance to these amino-alcohol containing compounds may be in part attributed to PfPgh-1 (a protein which encoded by *pfmdr1* and localized to the food vacuole membrane) [Foley and Tilley, 1997], and the analogous lipophilicity of the compounds.

Compounds **7** and **9** display over 10- and 50-fold higher activity than that of totarol in the K1 strain and over 10- and 100-fold higher cytotoxicity respectively. There is therefore a significant increase in toxicity which may contribute to the observed increase in antiplasmodial activity, although the difference in activity between the D10 and K1 strains does suggest some selectivity in the action of compounds **7** and **9**.

Compound 8

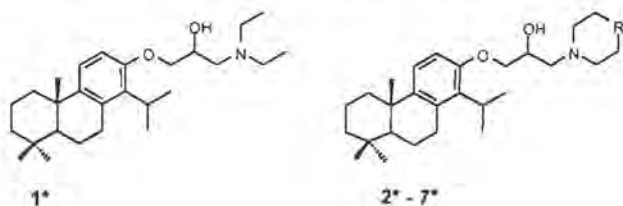


Compound **8** has better activity than that of totarol. It has a resistance index of 1.19, indicating that it has similar activity in both sensitive and resistant strains. Compound **8** is

also highly selective ($S_I = 10$). Its IC_{50} values are fairly high in comparison to the other derivatives synthesized and may be due to the absence of a protonatable nitrogen. Due to its weaker activity, **8** was not investigated further.

For comparative purposes, Table 5.3.1. below shows the IC_{50} values of the tertiary β -amino alcohol derivatives of totarol synthesized by Clarkson *et al.* (2003b).

Table 5.3.1.: IC_{50} values of tertiary β -amino alcohol totarol derivatives [Clarkson *et al.*, 2003b]; $R_I = IC_{50} (K1)/IC_{50} (D10)$; $S_I = IC_{50} (CHO)/IC_{50} (K1)$.



Compound	R	$IC_{50} (\mu M)$			R_I	S_I	
		D10	K1	CHO		D10	K1
1*	-	0.61	0.63	7.73	1.04	12.67	12.21
2*		1.4	0.9	94.19	0.64	67.28	104.89
3*		2.56	1.81	105.44	0.71	41.19	58.13
4*		3.25	0.9	>186	0.23	>57	>207
5*		1.62	1.01	>182	0.62	>112	>180
6*	O	2.07	0.56	5.61	0.27	2.71	10.04
7*	CH ₂	1.08	0.75	2.99	0.70	2.77	4.00

Figure 5.3.1. depicts all the β -amino alcohol derivatives whose antiplasmodial activities are compared in Figure 5.3.2.

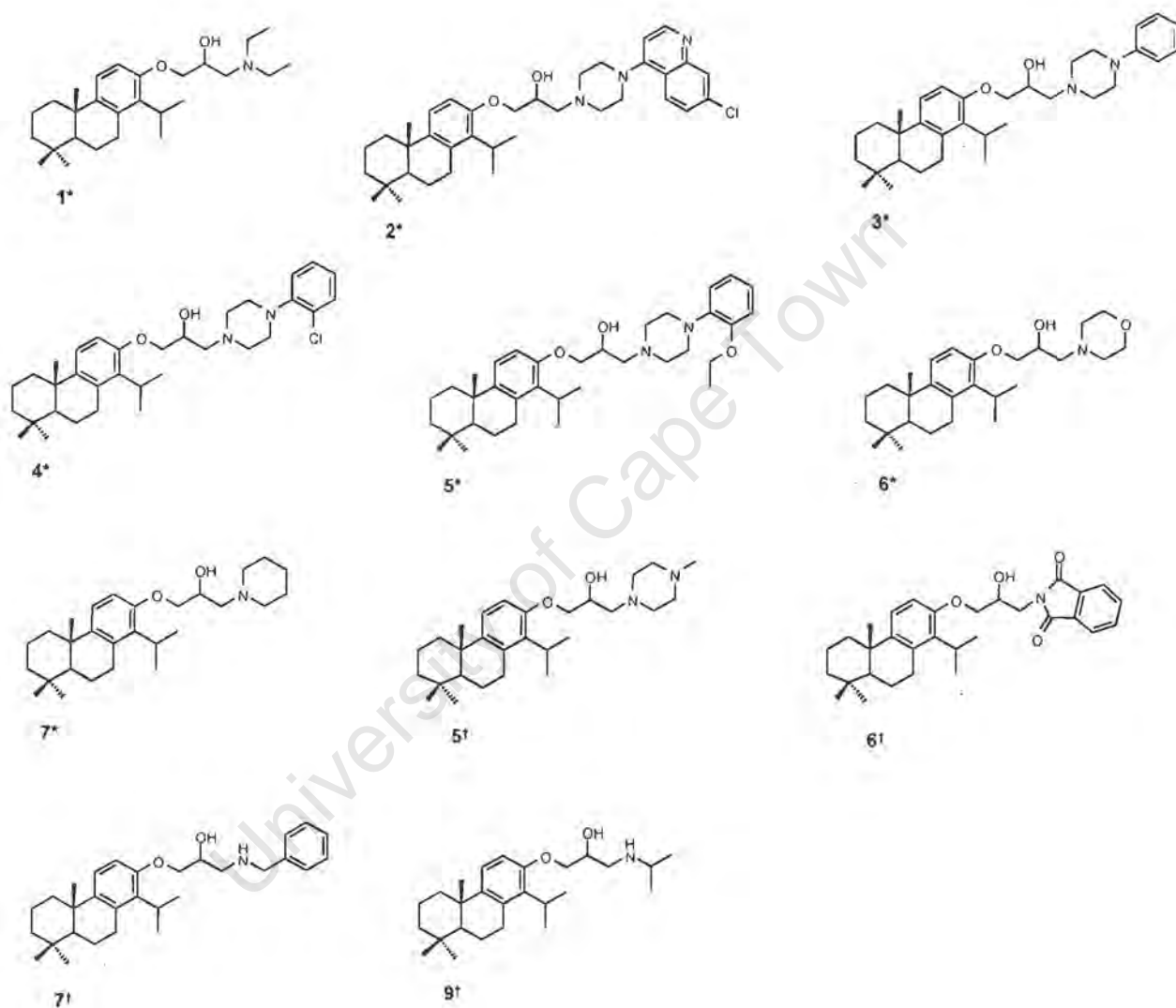


Figure 5.3.1.: Structure summary of β -amino alcohol totarol derivatives

* Compounds 1-7 synthesized by Clarkson *et al.* (2003b).

† Compounds 5, 6, 7 and 9 synthesized in this project.

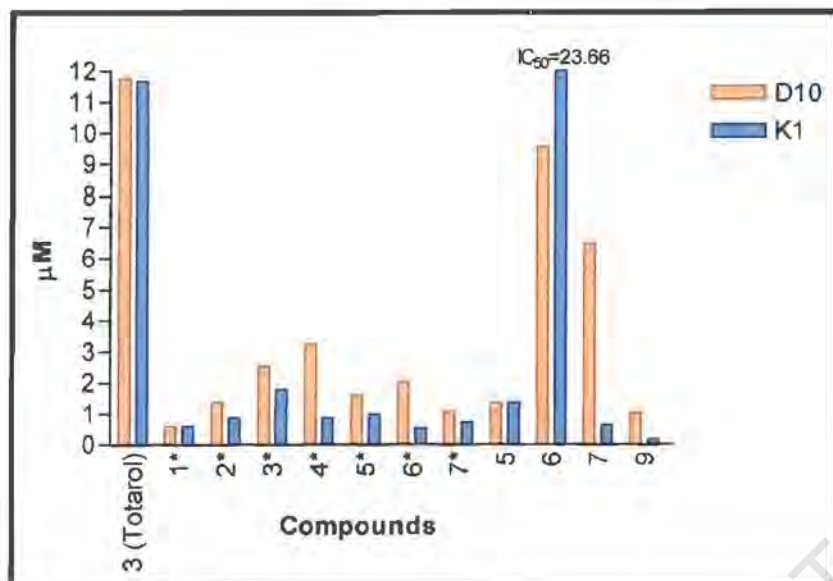


Figure 5.3.2.: Comparison of the IC₅₀ values of all the β -amino alcohol derivatives.

All β -amino alcohol derivatives have better activity than that of totarol (except **6** in K1 strain - pg 73). The majority of the β -amino alcohol derivatives have resistant indices that vary around 1. The exceptions are **4***, **6***, **7** and **9**, all of which are most active in K1, and **6**, which is more active in D10. Comparison of compounds allows predictions to be made about the structure-activity relationships. Comparison of **6*** and **7*** indicates that substitution of the O with a CH₂ moiety decreases activity in the K1 strain. Another possibility is that the mechanisms by which chloroquine resistance is conferred result in increased susceptibility to compounds **4***, **6***, **7** and **9**.

Compounds **2***-**5*** and compound **5** have similar structures in that they all possess a piperazine moiety forming the amino portion of the β -amino alcohol. Their IC₅₀ values are also comparable and do not vary greatly from sensitive to resistant strains (with the exception of **4***). This moiety may therefore play some role in the mode of action of these compounds, but addition of a 2-chlorophenyl group (**4***) greatly enhances activity in the resistant strain.

Compound **1*** has a greater selective antiplasmodial activity than compound **5**, indicating that a second protonatable nitrogen decreases selectivity.

Compounds **1***, **6*** and **7*** all have similar activity in the K1 strain. All of these compounds are cytotoxic. Compounds **6*** and **7*** however, have better activity in the K1 strain indicating that there is a level of selectivity towards the parasite. The difference in activity in the K1 strain between these compounds and all the other β -amino alcohol derivatives implies that a piperazinyl moiety may not be necessary for activity, and a bulky side-chain attached to the piperazine moiety perhaps reduces the compounds' activity by interfering with binding to the target molecule.

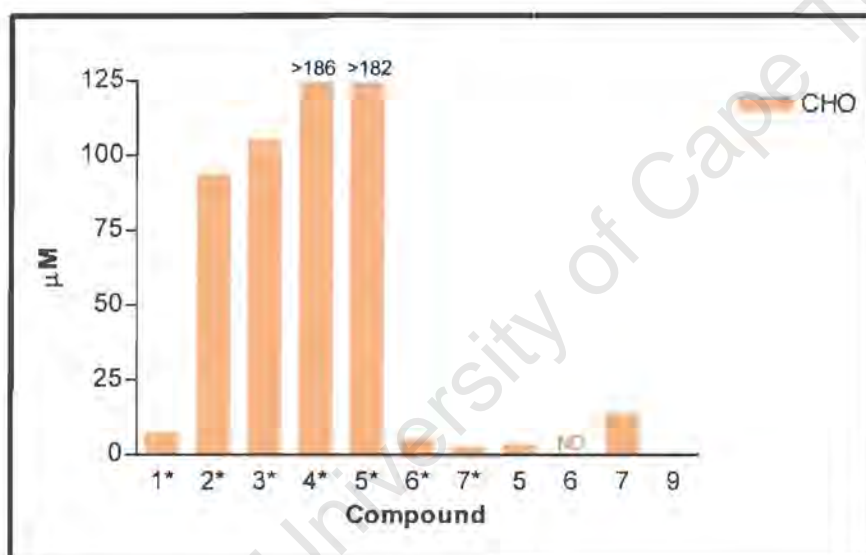


Figure 5.3.3.: Comparison of the cytotoxicity of all the β -amino alcohol derivatives.

(ND = Not determined as activity was not greater than that of totarol).

See pg 79 for structures

Cytotoxicity and selectivity indices of the compounds were generally found to correlate. The less basic the atoms in the side chain, particularly the β -nitrogen, the more selective (and less cytotoxic) the compound. The two most selective compounds are **4*** and **5***. Both of these compounds have β -N attached to an electron-withdrawing aromatic group. The

electron pair on the N is therefore delocalized into the aromatic ring and hence not entirely available for interactions with other molecules or cellular components.

Table 5.3.2: Table showing fold increase in antiplasmodial activity and cytotoxicity of β -amino alcohol derivatives with respect to totarol (See pg 79 for structures of compounds).

Compound	Fold increase in activity	Fold increase in cytotoxicity
5	8.4	45.7
7	18	11.8
8	1.2	1.8
9	68.8	133.2
1*	18.5	22.05
2*	13	1.8
3*	6.4	1.6
4*	13	<0.9
5*	11.6	<0.9
6*	20.9	30.4
7*	15.6	57.0

In general an increase in activity is accompanied by a substantial increase in cytotoxicity, except with compounds **8** and **2*-5***, indicating that the β -amino alcohol substituent imparts toxicity and hence non-selective antiplasmodial activity. Compounds **2*-5*** possess tertiary electron-poor β -nitrogen atoms and bulky aromatic side chains indicating that these structures may be important in conferring selectivity. Compound **8** is not a β -amino alcohol, although the fold increase in its antiplasmodial activity and cytotoxicity are similar, indicating that the hydroxyl moiety may also play a role in the toxicity.

5.4. Summary and Conclusion

In general, the addition of a β -amino alcohol side chain greatly increases the cytotoxicity and therefore is most likely responsible for the increased antiplasmodial activity of the totarol derivatives.

Compounds **5**, **7** and **9** displayed the highest antiplasmodial activities out of the compounds synthesized in this project. Comparison with compounds **6** and **8** leads to the assumption that a protonatable nitrogen is necessary for activity. This activity however, does not appear to be selective, as selectivity indices tend to decrease with increasing basicity of the β -nitrogen.

Comparison of the derivatives' fold increase in antiplasmodial activity and cytotoxicity relative to tatarol suggests that an electron-poor nitrogen attached to a bulky aromatic side chain imparts selective activity and that the hydroxyl moiety may play a role in the observed toxicity.

Chapter 6

***Effect of Replacing the Totarol Backbone on
In Vitro Antiplasmodial Activity and
Cytotoxicity of Selected Compounds***

6.1. Introduction

Totarol is an expensive compound, both to purchase and synthesize. It was therefore decided to determine whether replacing the totarol backbone would yield analogues with similar if not superior selective antiplasmodial activities, as well as determining the role of the totarol backbone in the antiplasmodial activity.

6.2. Results

The totarol backbone of selected derivatives (Figure 6.2.1.) was replaced with simple aromatic groups such as, phenol, 1-naphthol and 2-naphthol (Figure 6.2.2.). Compound **9** was also compared with its 1-naphthol derivative, **18**. The *in vitro* antiplasmodial activities of the analogues are shown in Table 6.2.1..

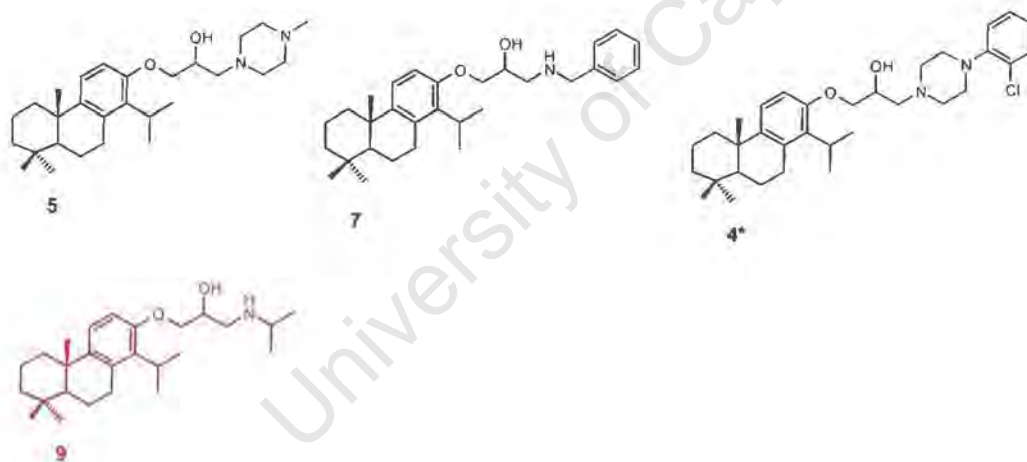


Figure 6.2.1.: Derivatives selected for totarol backbone replacement.

(Note: Although compound **9** was synthesized for investigation into its effect on erythrocyte invasion, it will be discussed with reference to the effect of totarol substitution with 1-naphthol on antiplasmodial activity and cytotoxicity).

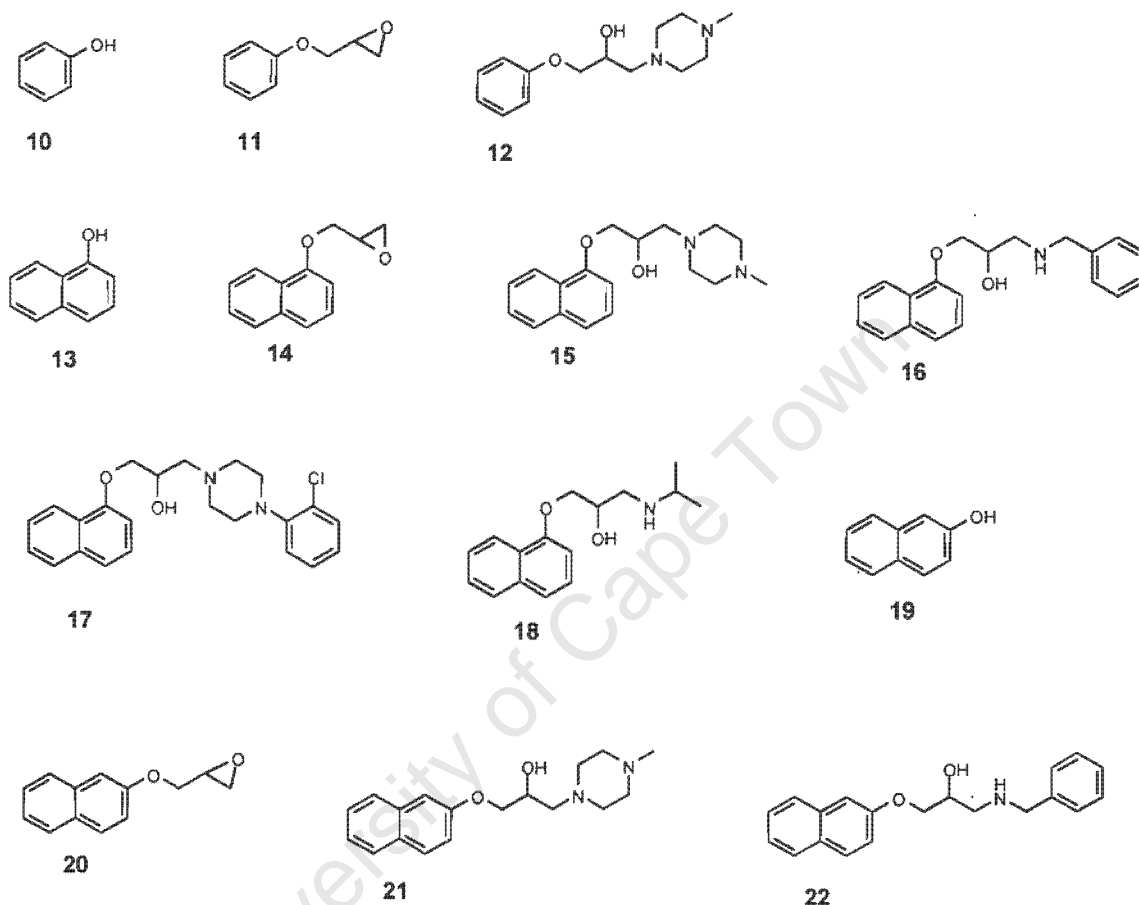


Figure 6.2.2: Structure summary of analogues 10-22.

Table 6.2.1.: Summary of the IC₅₀ values of totarol analogues as well as the calculated R_i and S_i values [R_i = IC₅₀ (K1)/IC₅₀ (D10); S_i = IC₅₀ (CHO)/ IC₅₀ (K1)].

Compound	IC ₅₀ µg/ml (µM)			R _i	S _i	
	D10	K1	CHO		D10	K1
3 (Totarol)	3.38 ± 0.72 (11.78 ± 2.53)	3.35 ± 0.22 (11.69 ± 0.78)	48.79 ± 0.96 (170.46 ± 3.35) [Clarkson <i>et al.</i> , 2003]	0.99	14.5	14.6
10	>100	ND	ND	-	-	-
11	11.57 ± 2.91 (77.04 ± 19.38)	ND	ND	-	-	-
12	>100	15.07 ± 3.53 (60.18 ± 14.10)	>100	>0.2	>1	>6.6
13	8.15 ± 0.48 (56.51 ± 3.32)	ND	ND	-	-	-
14	25.82 ± 1.90 (128.96 ± 9.49)	ND	ND	-	-	-
15	12.56 ± 0.59 (41.81 ± 1.97)	2.17 ± 0.30 (7.23 ± 0.98)	78.51 ± 3.88 (261.35 ± 12.92)	0.17	6.2	36.1
16	1.59 ± 0.15 (5.18 ± 0.48)	0.23 ± 0.01 (0.73 ± 0.02)	2.54 ± 0.17 (8.27 ± 0.56)	0.14	1.6	11.2
17	>100	ND	ND	-	-	-
18	9.77 ± 1.14 (37.68 ± 4.40)	2.64 ± 1.09 (10.19 ± 4.20)	>100	0.27	>2.6	>37.8
19	16.58 ± 1.98 (115.00 ± 13.73)	ND	ND	-	-	-
20	37.47 ± 6.70 (239.97 ± 42.91)	ND	ND	-	-	-
21	12.90 ± 0.23 (42.94 ± 0.76)	2.47 ± 0.84 (8.21 ± 2.79)	44.67 ± 2.96 (148.70 ± 9.84)	0.19	3.5	18.1
22	2.08 ± 0.81 (6.77 ± 2.64)	4.16 ± 1.32 (13.53 ± 4.29)	37.93 ± 6.63 (123.40 ± 21.58)	2.0	18.2	9.1

ND = Not determined (Activity not greater than that of totarol).

6.3. Discussion

6.3.1. Phenol substitution

Figure 6.3.1.1. depicts the selected totarol derivative, **5** and its corresponding phenyl analogue.

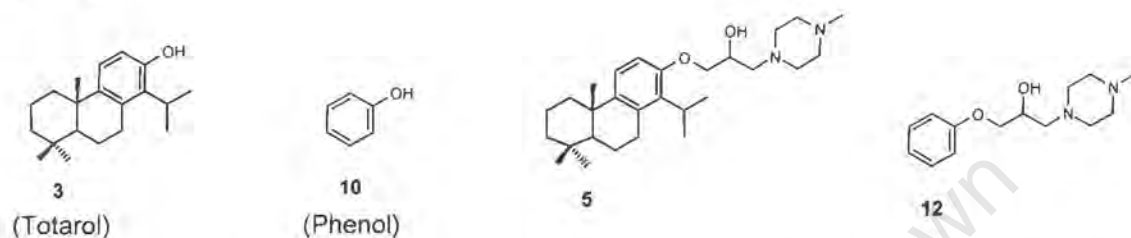


Figure 6.3.1.1.: Summary of parent compounds, totarol derivative and phenyl analogue.

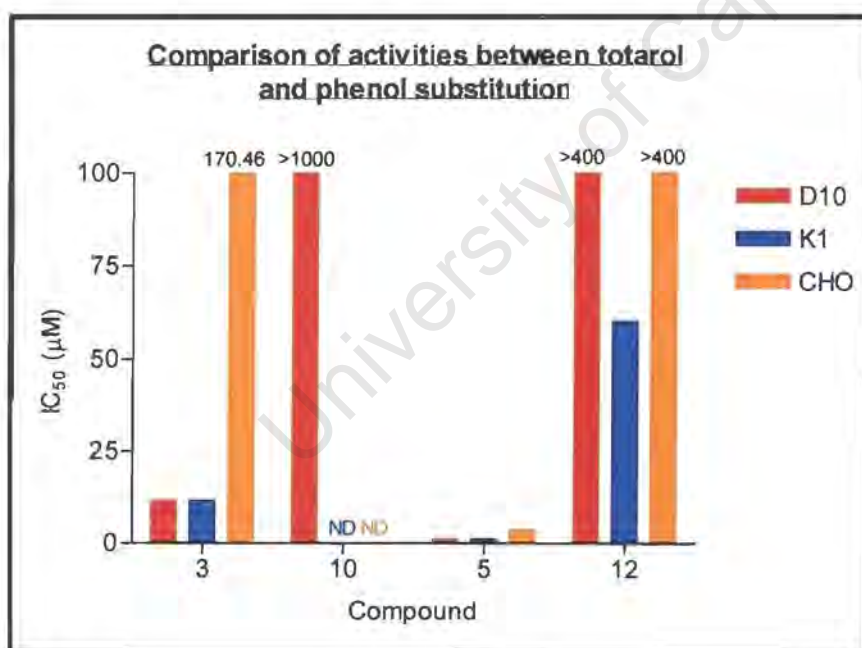


Figure 6.3.1.2.: Comparison of activities between totarol (**3**) and phenol (**10**) as well as compounds **5** and **12**.

(ND = Not determined as activity was not greater than that of totarol).

As can be seen in Figure 6.3.1.2., the substitution of the totarol backbone with a phenyl group completely abolishes activity.

6.3.2. 1-Naphthol substitution

Figure 6.3.2.1. depicts selected totarol derivatives and their corresponding 1-naphthyl analogues.

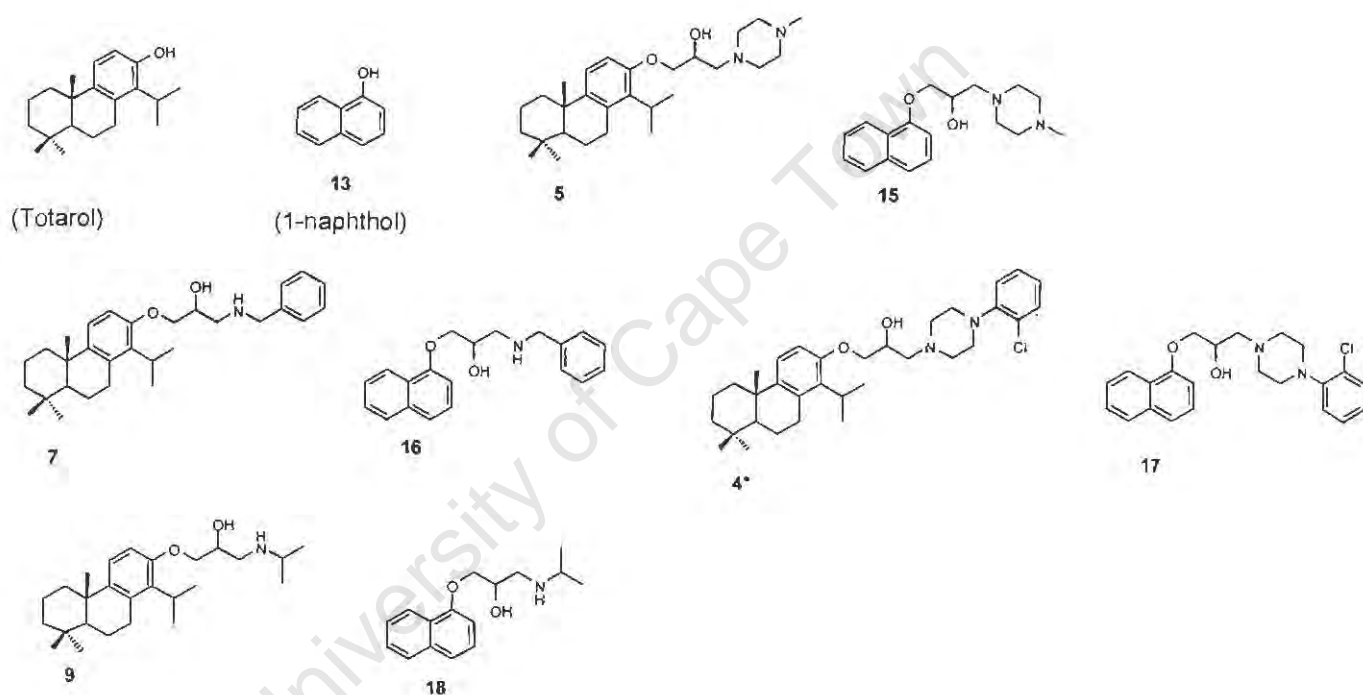


Figure 6.3.2.1.: Summary of parent compounds, totarol derivatives and 1-naphthyl analogues.

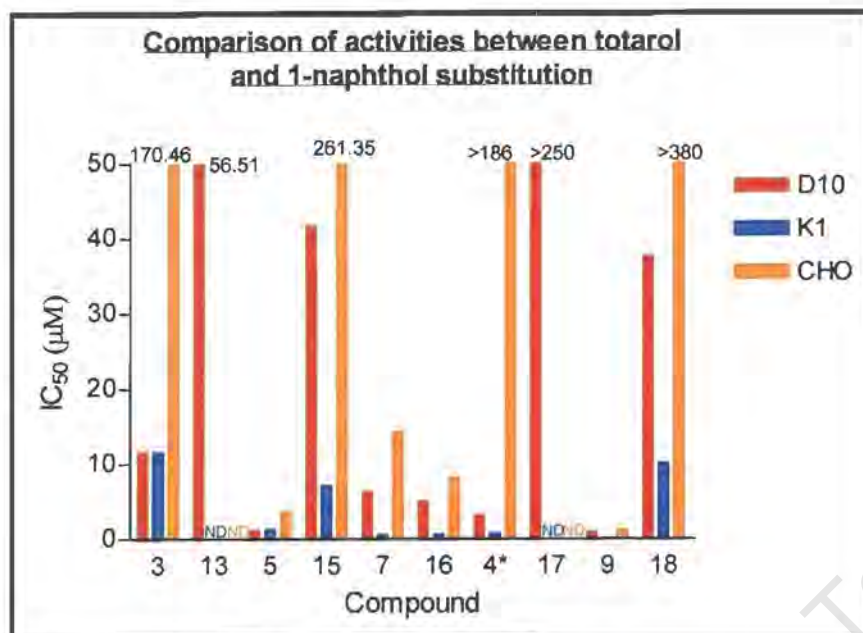


Figure 6.3.2.2.: Comparison of activities between totarol (3), 1-naphthol (13), totarol derivatives (5, 7, 4* and 9) and 1-naphthyl analogues (15, 16, 17, 18).

(ND = Not determined as activity was not greater than that of totarol).

The privileged compound totarol (3) is again much more active than the simpler aromatic, 1-naphthol (13). Replacement of the totarol backbone in compounds 5, 4* and 9 drastically decreases activity. The behaviour of 9 (i.e. more active in the K1 strain) is however conserved in compound 18. One may therefore attribute this increased activity in K1 to the isopropylamine side chain. The activity of compound 5 is similar in both the D10 and K1 strains, while its analogue, 15 does not display similar behaviour as its activity is far greater in the K1 strain. This suggests that the two compounds (5 and 15) have different target(s). In the case of 4*, activity was completely abolished at the maximum concentration tested, indicating that the totarol backbone is solely responsible for the observed activity of 4*.

Replacement of the totarol scaffold in 7 does not abolish the antiplasmodial activity or toxicity. Compound 7 and its 1-naphthyl analogue, 16 have comparable activity, toxicity and behaviour (i.e. increased activity in K1). The antiplasmodial activity of compound 16 relative to all the other 1-naphthyl analogues seems to correlate with its cytotoxicity although the significant (7-fold) difference in activity between the D10 and K1 strains

implies that this compound is somewhat selective. Compound **16** is 1.2-, 0.9- and 1.7-times more active/toxic than **7** in D10, K1 and CHO respectively. Comparison of the activity of compounds **7** and **16** indicates that the benzylamine side-chain is responsible for the observed toxicity and somewhat selective antiplasmodial activity. Replacement of the totarol backbone does however seem to increase toxicity of this compound.

6.3.3. 2-Naphthol substitution

Figure 6.3.3.1. depicts the selected totarol derivatives and their corresponding 2-naphthyl analogues.

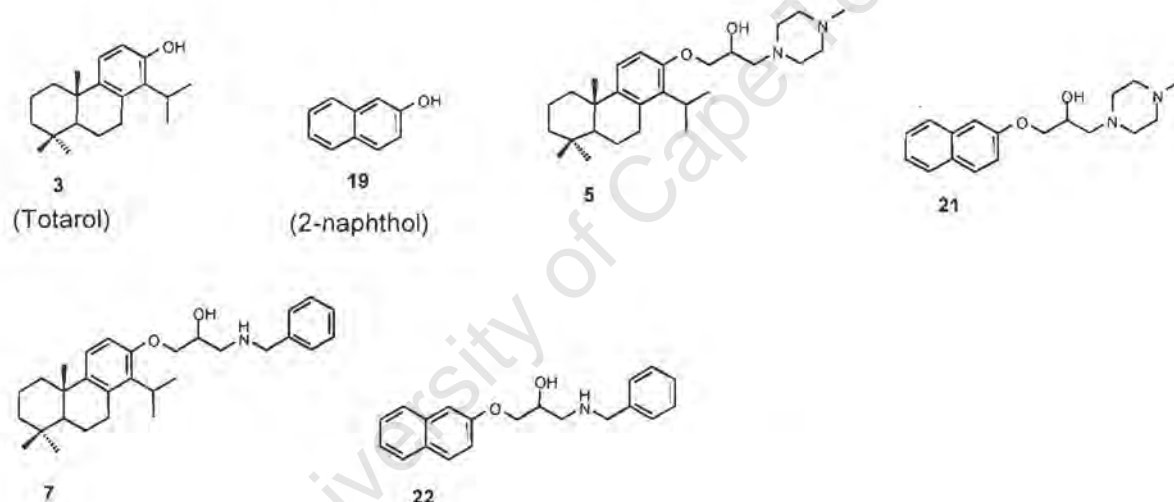


Figure 6.3.3.1.: Summary of parent compounds, totarol derivatives and 2-naphthyl analogues.

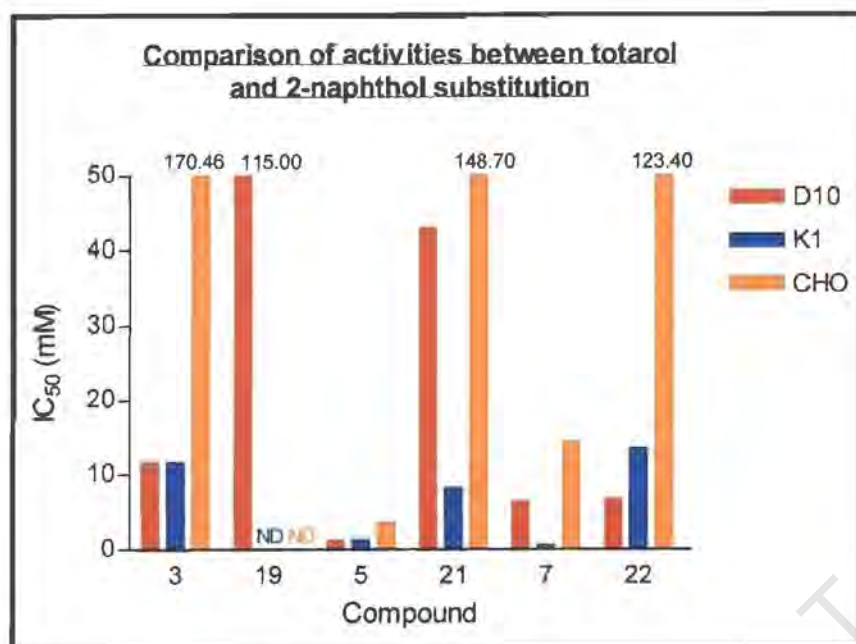


Figure 6.3.2.2.: Comparison of activities between totarol (**3**), 1-naphthol (**19**), totarol derivatives (**5** and **7**) and 2-naphthyl analogues (**21** and **22**). (ND = Not determined as activity was not greater than that of totarol).

Totarol (**3**) is again much more active than 2-naphthol (**19**). The replacement of the totarol backbone in compound **5** decreases antiplasmodial activity although **21** is much less toxic to CHO cells. Compounds **5** and **21** do not display similar behaviour and **21** is much more active in K1. The replacement of the totarol scaffold in **7** however does not abolish the activity, and compound **7** and its 2-naphthyl analogue, **22** have comparable activity in the D10 strain. The behaviour evident in compound **7** (i.e. increased activity in K1) is not mimicked in **22**. The replacement of totarol in **7** decreases the cytotoxicity by a factor of 8.

6.4. Summary and Conclusion

In general, totarol derivatives were much more active and cytotoxic than the phenyl, 1-naphthyl or 2-naphthyl analogues. This was expected as totarol, having been isolated from a natural source, is a privileged structure and hence it is more likely to possess biological activity. However, 1- and 2-naphthyl analogues with a benzylamine side-chain (**16** and **22**)

displayed similar activity to their tatarol counterparts. One can therefore assume that, for compounds other than **16** and **22** where the benzylamine appears to be responsible for the activity, the tatarol backbone is necessary for maximal receptor(s) binding. It is also possible that the tatarol derivatives and their respective analogues have different target(s) and mechanism(s) of action, especially those displaying behavioural differences, thereby accounting for the observed differences in activity and toxicity.

The tatarol backbone, in most cases, seems to impart toxicity and therefore influence selectivity. Tatarol, as mentioned, is a privileged structure, and may therefore display promiscuous binding, resulting in toxicity. Compounds **7** and **16** had similar high cytotoxicities, while compound **22** in comparison had considerably decreased toxicity. This indicates that the backbone may play a significant role in the toxicity, although the position of a side-chain, and indeed the side-chain itself may influence the toxicity and selectivity of the compound.

All analogues are racemic mixtures. This may well influence both the observed antiplasmodial activities and cytotoxicities as one enantiomer may antagonize the action of the other or possess unselective activity.

Chapter 7

Effect of Totarol and its Derivatives on Erythrocyte Morphology and Lysis

University of Cape Town

7.1. Introduction

The growth of *P. falciparum* is dependent on erythrocytic transport processes and hence drug effects on erythrocytes may influence parasite viability and growth [Kirk, 2001]. In general, amphiphilic compounds, lipophilic compounds and compounds which incorporate into membranes cause membrane modifications and hence shape changes [Ziegler *et al.*, 2002a and b]. As mentioned earlier, totarol has been shown to incorporate into erythrocytic membranes [Mateo *et al.*, 2000; Micol *et al.*, 2001]. Totarol derivatives may therefore inhibit parasite growth indirectly by incorporating into the erythrocytic membranes and hence may not be true antiparasmodial mediators.

7.2. Results and Conclusions

The effect of a range of concentrations of totarol, its derivatives and selected analogue (compounds 3, 5-7, 9 and 16) on erythrocytic lysis was determined (as described in 4.5.). The structures of the compounds are shown in Figure 7.2.1. and the results in Figure 7.2.2..

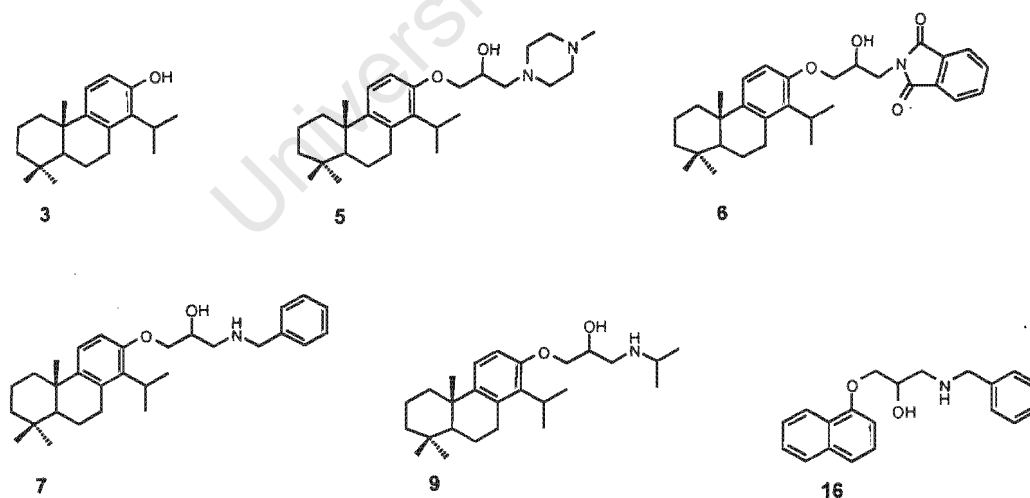


Figure 7.2.1.: Compounds tested for their lytic and morphological effects on erythrocytes.

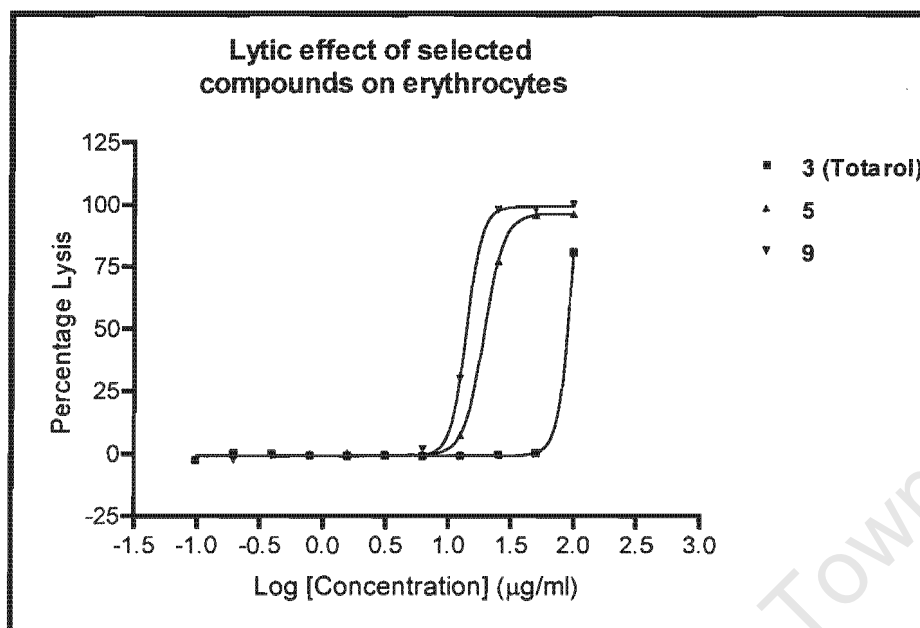


Figure 7.2.2: Graph showing the lytic effect of different concentrations of totarol derivatives.

Compounds **6**, **7** and **16** do not cause lysis at the selected concentrations (highest concentration was 100 μg/ml).

None of the totarol derivatives, nor compound **16** have apparent antiplasmodial effects due to erythrocytic lysis.

Totarol, its derivatives **5**, **6**, **7**, **9** and the analogue possessing the highest antiplasmodial activity, **16** (structures shown in Figure 7.2.1.) were all examined for their effects on erythrocytic shape at sub-lytic concentrations (as described in 4.6.). The results are shown in Figure 7.2.3..

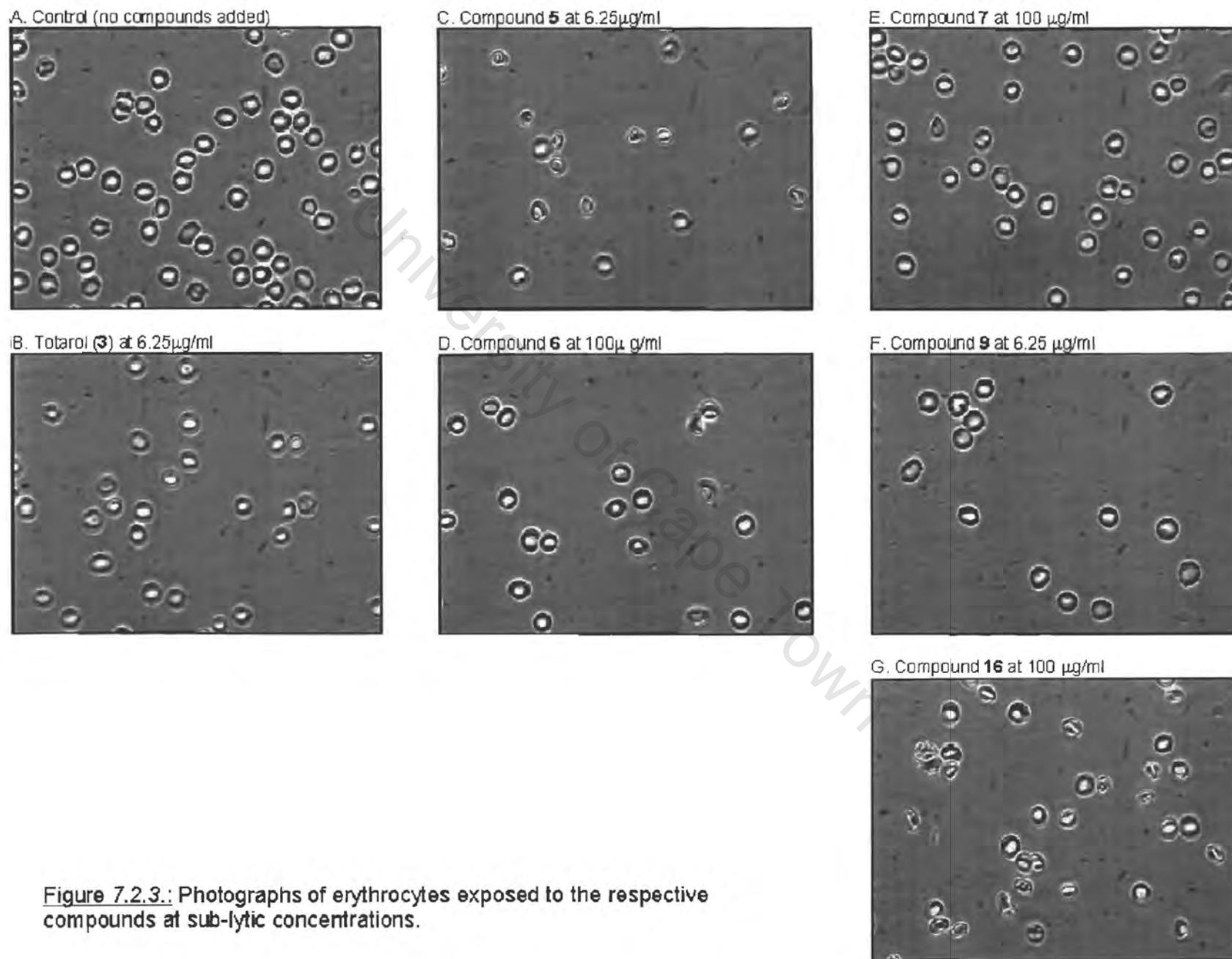


Figure 7.2.3.: Photographs of erythrocytes exposed to the respective compounds at sub-lytic concentrations.

Compounds **5** (6.25µg/ml) and **16** (100µg/ml) caused modification of erythrocytic shape, although the morphological changes did not correlate with their respective activities, although this is a qualitative study and only gross effects are observed. Detailed analysis and observations would therefore have to be performed using electron microscopy.

7.3. Summary

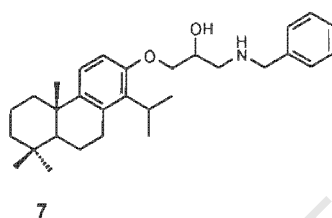
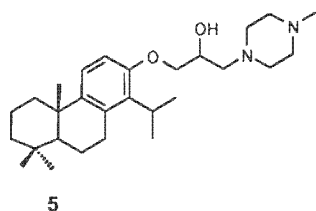
The effect of potential lead antiplasmodial compounds on erythrocytic lysis and shape is an important factor to address when considering if the observed activity is selective and indeed truly antiplasmodial. The compounds tested had no effect on erythrocytic lysis and morphology at concentrations corresponding to their respective antiplasmodial activities. The activity displayed by these compounds is therefore specific for the parasite and not an observed anomaly due to toxic effects on the erythrocyte.

Chapter 8

***Effect of Selected Derivatives on CQ
Accumulation and Potentiation***

8.1. Introduction

An alternative strategy in combating resistance is to develop compounds which can reverse resistance of *P. falciparum* to antimalarial drugs. Chemosensitizers act synergistically with the antimalarial to which resistance has developed and in so doing potentiate its activity to a level approaching that obtained in a sensitive strain.



Compounds **5** and **7** display general characteristic structural elements similar to those possessed by compounds which are able to modulate multidrug resistance in cancer cells. The majority of the compounds which act as resistance reversers have been noted to be lipid soluble, have two planar aromatic rings, a cationic charge and a protonatable tertiary nitrogen [Beck, 1990; Rasoanaivo *et al.*, 1996; Zamora *et al.*, 1988]. Both compounds **5** and **7** are composed of a diterpene-like totarol portion, which is lipid soluble and contains a planar aromatic ring. The *N*-methylpiperazine portion of **5** has two protonatable tertiary nitrogens. Compound **7** does not possess a tertiary nitrogen, but the secondary NH group may become protonated in an appropriate acidic medium resulting in a cationic charge. It is therefore possible that these compounds could modulate CQ resistance.

In order to determine whether or not the respective compounds had the potential to increase the accumulation of CQ, an uptake experiment using tritiated chloroquine (³H-CQ) on a CQ resistant (K1) strain was performed as described in section 4.3.. Resistant parasite strains accumulate less CQ than sensitive strains [Fitch, 1970]. Compounds which are able to increase CQ accumulation in a resistant strain are therefore thought to have the potential to act as resistance reversal agents. The results obtained from the

uptake would give an indication as to whether or not the compounds possess any chloroquine modulation potential, as well as showing the optimum concentration at which to perform a resistance reversal assay (i.e. a sub-lethal concentration at which there is 100% parasite viability and maximal CQ uptake). CQ potentiation is not however totally reliant on a compound increasing CQ accumulation and similarly a compound which increases CQ accumulation may not possess CQ potentiating ability [Bray and Ward, 1998; Chibale *et al.*, 2003; Verdier *et al.*, 1985]. The effect of the compounds on CQ potentiation was performed as described in section 4.4.. Response modification indices (RMI) are calculated in order to ascertain the CQ modulating activity of the respective compounds. A RMI of greater than 1 indicates antagonistic behaviour between the potential resistance modulator and CQ, while a RMI below 1 indicates synergistic behaviour [Gerena *et al.*, 1992].

Verapamil is a well established resistance modulator which increases CQ accumulation [Martin *et al.*, 1987]. It was therefore used as a positive control in both the ^3H -CQ accumulation and CQ potentiating experiments.

8.2. Effect of compound 5 on CQ accumulation and potentiation.

8.2.1. Effect of compound 5 on CQ accumulation

Figure 8.2.1.1. shows the effect of compound 5 on the uptake of ^3H -CQ accumulation.

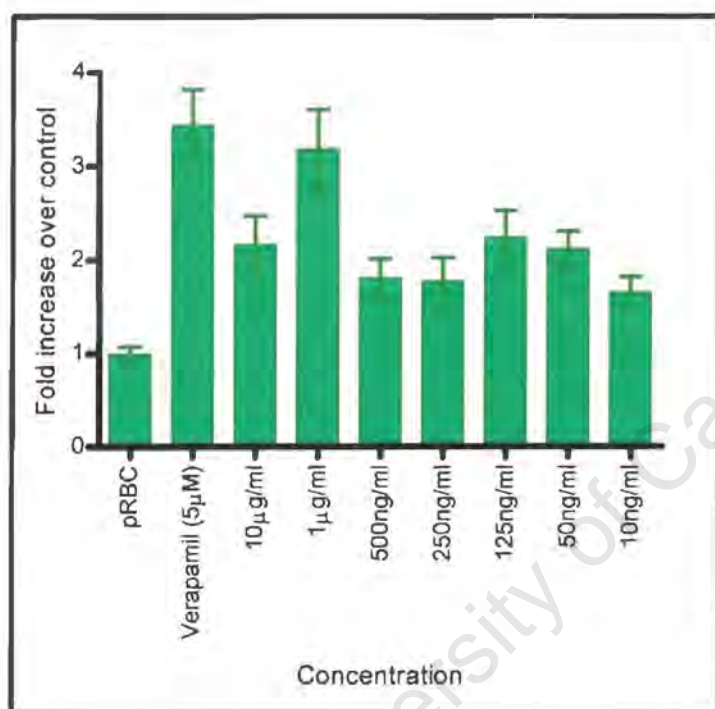


Figure 8.2.1.1.: The effect of 5 on ^3H -CQ accumulation in the CQ resistant K1 strain.

Table 8.2.1.1.: Increase in CQ accumulation caused by compound 5.

Concentration of 5	Fold increase over pRBC control	P value	Significantly different to pRBC accumulation
10 μg/ml	2.168	0.0034	Yes
1 μg/ml	3.185	0.0002	Yes
500 ng/ml	1.808	0.0030	Yes
250 ng/ml	1.774	0.0145	Yes
125 ng/ml	2.243	0.0012	Yes
50 ng/ml	2.119	< 0.0001	Yes
10 ng/ml	1.651	0.0042	Yes

A significant increase in chloroquine accumulation was noted within the concentration range between 10 μ g/ml to 10ng/ml. Interestingly, little difference in the accumulation was seen in the concentration range of 500ng/ml to 10ng/ml. A 50-fold dilution therefore had little effect on chloroquine accumulation. The constant level of the graph may indicate that there are a limited number of specific target molecules (e.g. chloroquine or proton influx or efflux pumps) to which **5** is binding. At a concentration of 10ng/ml all the proteins are bound and therefore a further increase in compound **5** has little effect on accumulation. The IC₅₀ value of **5** is 0.67 μ g/ml for the resistant (K1) strain. Above this IC₅₀ value further increase in accumulation is observed. This increase may be due to unspecific action as the compound is highly toxic at these concentrations. The decrease in accumulation observed at 10 μ g/ml is most probably due to lysis of the erythrocyte caused by the high concentrations of **5** (chapter 7).

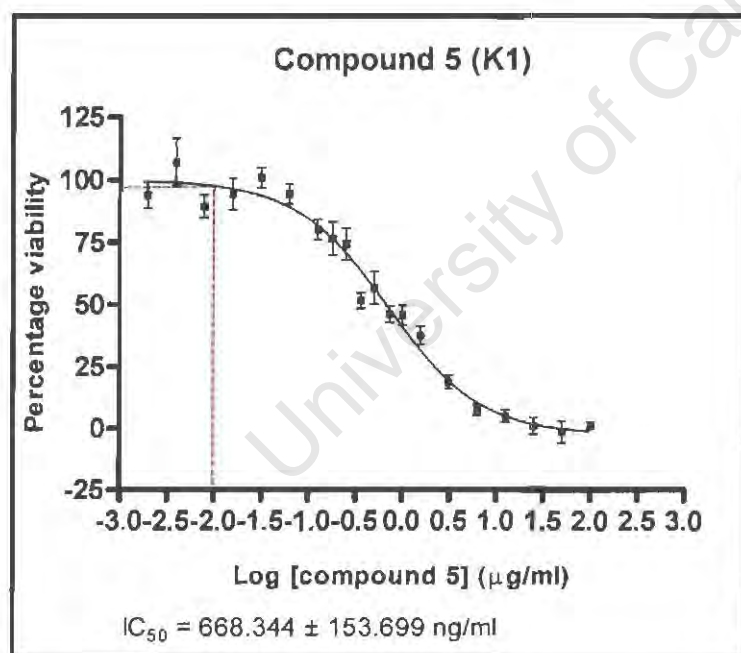


Figure 8.2.1.2.: Dose response curve of compound **5**.

The largest significant uptake of ^3H -CQ was seen at a concentration of 1 μ g/ml of compound **5**. The resistance reversal assay would therefore ideally be performed at this concentration. However, it is clear from the dose response curve (Figure 8.2.1.2.) that at a concentration of 1 μ g/ml, compound **5** possesses significant intrinsic antiparasmodial activity.

As shown by the dotted line on the above dose response curves of **5**, the concentration at which parasite viability is closest to 100% is 10 ng/ml ($\log[10\text{ng/ml}] = -2.0$). The resistance reversal assay was therefore performed at 10ng/ml as at this concentration there is close to 100% parasite viability and a high CQ uptake.

8.2.2. Chloroquine potentiating effect of **5**

Figure 8.2.2.1. below shows the dose response curve of CQ alone and in combination with compound **5** and Verapamil (control). There is no evident shift, as is seen with verapamil, in the CQ+**5** curve when compared to the CQ curve. This indicates that **5** does not have CQ potentiating ability, although RMIs must be compared before conclusions can be made.

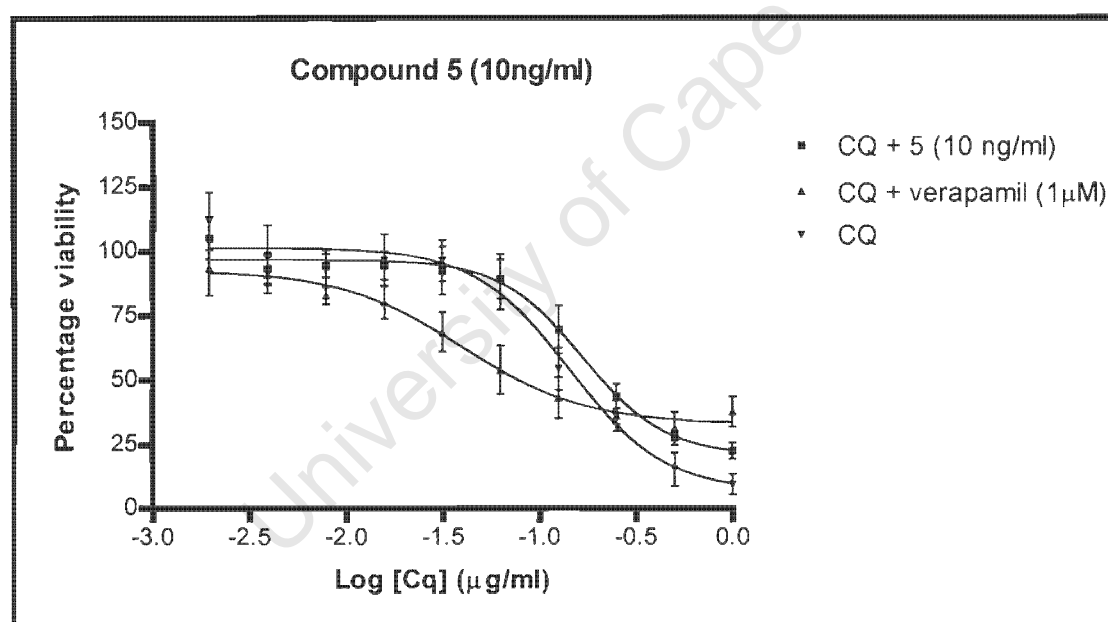


Figure 8.2.2.1.: Dose response curves of compound **5** + CQ, Verapamil + CQ and CQ.

Table 8.2.2.1.: RMIs obtained for verapamil and compound 5.

Compound	IC₅₀ CQ + compound (ng/ml)	RMI = IC₅₀ CQ + compound / IC₅₀ CQ
CQ	158.489	-
Verapamil (1μM)	78.343	0.49
Compound 5 (10ng/ml)	211.836	1.34

Compound **5**, although it increases CQ accumulation does not potentiate CQ action, as indicated by its RMI of 1.34. This indicates that CQ and **5** work antagonistically. Compound **5** may increase CQ accumulation, by a number of mechanisms such as;

- Alteration of vesicle pH causing a greater pH gradient,
- Inhibition or alteration of possible CQ efflux and/or influx mechanisms.

Compound **5** may antagonize CQ's action by preventing binding to its target molecule(s).

8.3. Effect of compound 7 on CQ accumulation and potentiation.

8.3.1. Effect of compound 7 on CQ accumulation

Figure 8.3.1.1. shows the effect of compound 7 on ^3H -CQ accumulation.

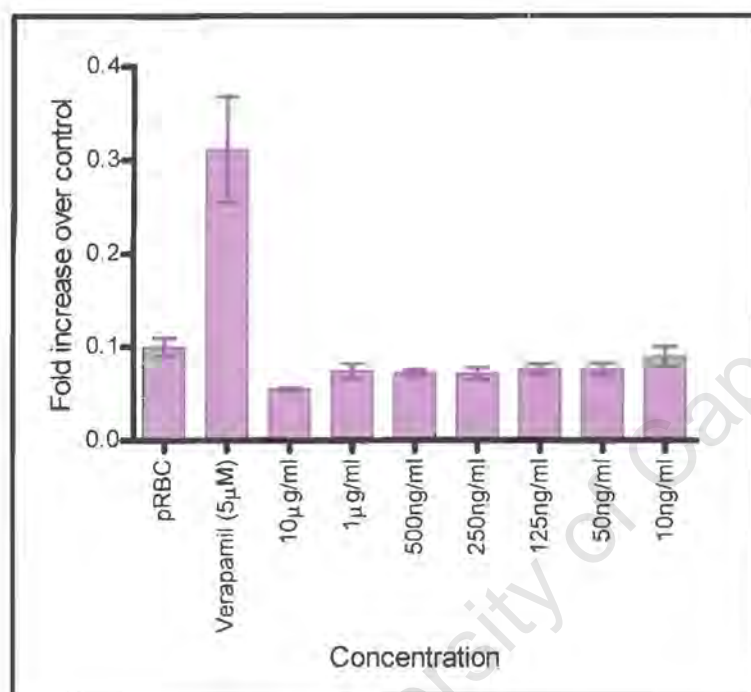


Figure 8.3.1.1.: The effect of 7 on ^3H -CQ accumulation in the CQ resistant K1 strain.

Compound 7 does not increase ^3H -CQ accumulation, and therefore does not influence the mechanisms by which CQ accumulation occurs. This however does not discard the possibility that 7 may still possess CQ potentiating ability, as it may act synergistically with CQ but not increase its accumulation.

8.3.2. Chloroquine potentiating effect of 7

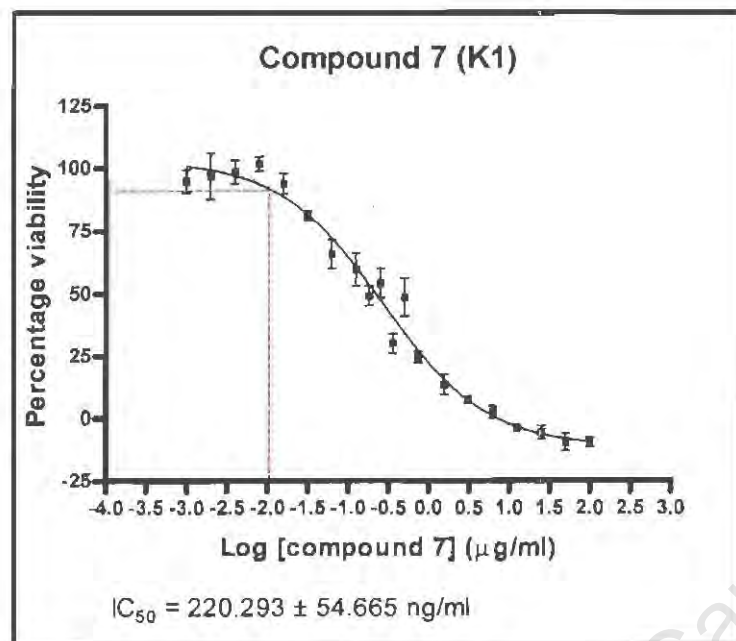


Figure 8.3.2.1.: Dose response curve of compound 7.

As shown by the dotted line on the above dose response curve of 7 (Figure 8.3.2.1.), the concentration at which parasite viability is closest to 100% is 10 ng/ml ($\text{log}[10\text{ng/ml}] = -2.0$). The assay was therefore performed at 10 ng/ml as compound 7 is sub-toxic at this concentration.

Figure 8.3.2.2. below shows the dose response curve of CQ alone and in combination with compound 7 and Verapamil (control). There is no evident shift in the CQ+7 curve when compared to the CQ curve. This indicates that 7 most likely does not have CQ potentiating ability.

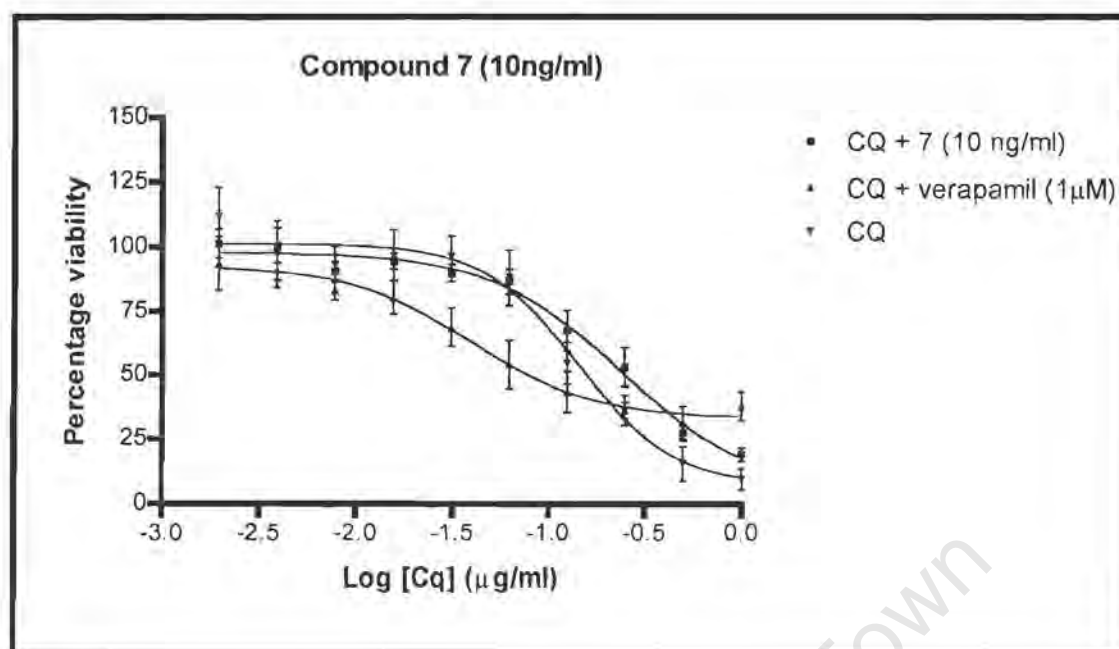


Figure 8.3.2.2.: Dose response curves of compounds **7** + CQ, Verapamil + CQ and CQ.

Table 8.3.2.1.: RMIs obtained for verapamil and compound **7**.

Compound	IC ₅₀ Compound + CQ (ng/ml)	RMI = IC ₅₀ compound + CQ/ IC ₅₀ CQ
CQ	158.489	-
Verapamil (1 μM)	78.343	0.49
Compound 7 (10ng/ml)	251.189	1.58

Compound **7** did not increase CQ accumulation and had a RMI of 1.58, indicating that CQ and **7** have antagonistic mechanisms of action.

8.4. Summary and Conclusion

Compound **5** increases ³H-CQ accumulation while compound **7** does not, although both compounds, at 10ng/ml, act antagonistically to CQ as indicated by their RMI values (RMI>1).

Chapter 9

Effect of Selected Compounds on Erythrocytic Invasion

9.1. Introduction

Parasite invasion of erythrocytes is a complex process consisting of several sequential events; (1) attachment of the merozoite to the erythrocyte; (2) positioning of the apical end of the merozoite towards the erythrocytic membrane; (3) formation of a tight junction; (4) internalization of the merozoite into a parasitophorous vacuole within the erythrocyte [Dvorak *et al.*, 1975].

Inhibition of invasion is an unique target for drug discovery and an understanding of the mechanism of invasion, as well as the discovery and development of compounds which prevent it are vital, both for increased knowledge of complex parasite biology, and also for new ways to combat malaria.

Compound **18** is the racemic form of the well known β -blocker (β -adrenergic receptor antagonist) propranolol, which has been shown to inhibit infection of erythrocytes by *P. falciparum*, as well as reducing the parasitaemia of *P. berghei* infections *in vivo* [Haldar *et al.*, 2003]. The similarity of the β -amino alcohol analogues to the common structural features associated with β -blockers and the results obtained by Haldar *et al.* (2003) prompted the investigation and comparison of the effect of compounds **16** and **18** and their respective totarol derivatives **7** and **9** on erythrocytic invasion.

9.2. Results

The effect of compounds **9**, **18** and **7**, **16** on erythrocytic invasion are summarized in Figure 9.2.1. and Table 9.2.1. below.

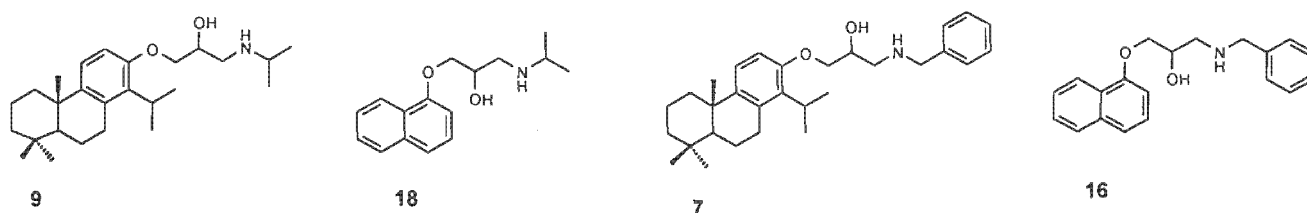


Figure 9.2.1.: Compounds tested for their ability to inhibit erythrocytic invasion.

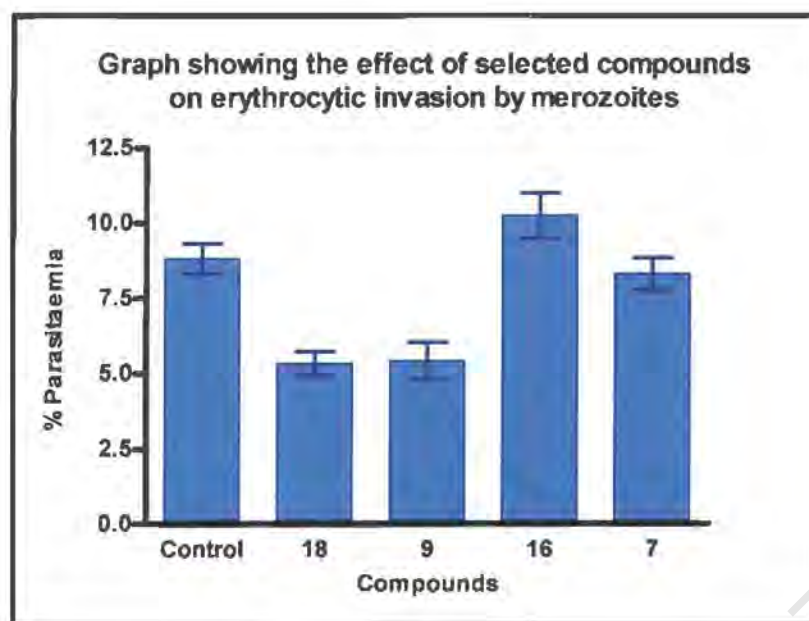


Figure 9.2.2: Effect of selected compounds on erythrocyte invasion.

Table 9.2.1.: % Parasitaemia's achieved by cultures treated with selected compounds

Compound	% Parasitaemia	% Parasitaemia relative to control	P Value	Significantly different to control
Control	8.83 ± 1.58	-	-	-
9	5.43 ± 1.87	61.5 ± 21.2	0.0005	Yes
18	5.35 ± 1.18	60.6 ± 13.4	<0.0001	Yes
7	8.31 ± 1.59	94.1 ± 18.0	-	No
16	10.24 ± 2.38	116.0 ± 27.0	-	No

Compound **18** (racemic form of propranolol) and its totarol derivative, **9** caused a decrease in parasitaemia relative to the control, while compounds **7** and **16** did not influence the parasitaemia.

9.3. Summary and Conclusion

It was found that compounds **9** and **18** (propranolol), tested at their respective IC_{50} values, approximately halved the parasitaemia relative to the control culture, while compounds **7** and **16** had no effect. This suggests that these two groups of compounds (isopropylamine-

and benzylamine-containing) have different mechanisms of action (in this period of the life-cycle¹), which are dependent on their side chains.

Propranolol (compound **18**) may be regarded as an established β -adrenergic receptor antagonist and therefore one can assume that it inhibits erythrocytic invasion by blocking the β 2-adrenergic receptors found on the erythrocyte's surface, thereby preventing G protein activation and possible cytoskeletal reorganization necessary for infection of the erythrocyte [Haldar *et al.*, 2003; Stryer, 2000]. The (+) stereoisomer of propranolol is inactive, although a racemic form of propranolol (compound **18**) was shown to inhibit infection by Haldar *et al.*, (2003) and in this study.

Compounds **9** and **18**, have very low resistance indices (R_i) indicating that they are more active in the K1 strain. With the compounds' effect on invasion taken into account, this low R_i may be due to other antiplasmodial effects of the compounds in the K1 strain.

¹ Inhibition of invasion may be one of the many effects of compounds **9** and **18**, although it is unlikely as 50% inhibition was seen at their respective IC_{50} values.

Chapter 10

Summary, Conclusions and Future Work

University of Cape Town

10.1. Summary

The addition of a β -amino alcohol side chain increases the activity of totarol in most cases and SAR studies revealed that a side chain containing a protonatable nitrogen may be necessary for activity, although a basic β -nitrogen appears to decrease selectivity.

The diterpene-like backbone of totarol appears to be important for antiplasmodial activity, as substitution with simpler aromatic groups decreased activity. Replacement of the backbone does however decrease toxicity. This is not surprising, as totarol is a natural product, and therefore, a privileged structure, presumably capable of binding to many proteinaceous molecules.

The activity of totarol, its derivatives and the most active analogue, **16**, was specifically antiplasmodial and not an observed false antiplasmodial effect due to conformational changes in the erythrocytes.

Compounds tested for resistance modulation potential were both shown to act antagonistically to CQ. Taken with these compounds' (and their analogues') antiplasmodial activity (which in most cases is greater in K1) it is possible to predict that these totarol derivatives and analogues have different, antagonistic mechanisms of action to that of CQ.

Two totarol derivatives and their respective analogues (containing benzylamine and isopropylamine side-chains) were tested for their effect on erythrocytic invasion. The compounds with the isopropylamine side-chain approximately halved the parasitaemia, relative to the control culture, at their respective IC_{50} values, suggesting that inhibition of invasion may possibly be one mechanism of action of these compounds.

10.2. Conclusion

A number of novel totarol derivatives and analogues were synthesized, characterized and tested for antiparasmodial activity. These derivatives add to the current information regarding structure activity relationships and possible modes of action. Compounds **5**, **7**, **9** and **16** hold potential as lead compounds provided that their toxicity and hence selectivity indices are improved.

10.3. Future Work

Expanded focused chemical libraries around the basic structures of the most promising compounds (**5**, **7**, **9** and **16**) using a variety of monomer building blocks could be generated in future studies in order to further investigate the structure-activity relationships. This will provide additional insight into the mechanism of action and toxicity of these totarol-based compounds. An important feature to investigate in terms of structure-activity relationships is the effect of pure enantiomers on antiparasmodial activity and toxicity. Often enantiomers have opposing activities, especially if the target is enzymatic (inactive enantiomer may act as an antagonist and the active enantiomer as an agonist). The activity of a racemic mixture is therefore a combined result of the activities of both enantiomer's, which would be less than the most active enantiomer's activity and more than the least active enantiomer's activity. Analogue synthesis resulted in racemic mixtures. Separation of these mixtures and the determination of the separate enantiomer's antiparasmodial activity may result in more selective and active analogues, as well as indicating whether the target(s) is enantioselective. Performing these studies on the separate enantiomers *in vitro* on various parasite strains and mammalian cell lines, as well as *in vivo* testing of compounds **5**, **7**, **9**, and **16** may provide better insight into structure-activity relationships and activity, thereby aiding in identifying possible lead compounds.

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Appendices

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Appendix 1

Table showing the molecular weight and clog P values of compounds	128
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Appendix 2

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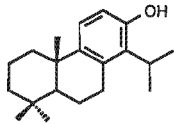
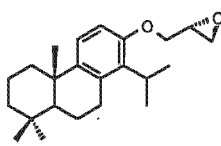
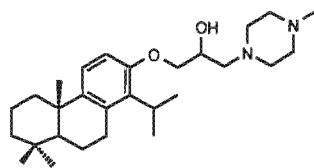
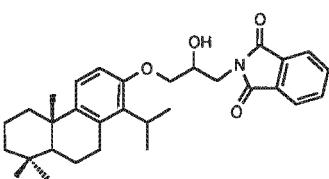
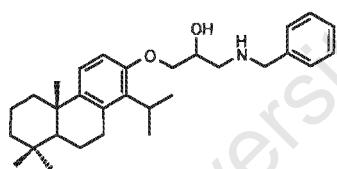
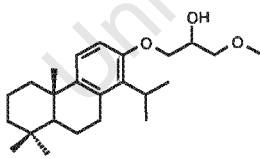
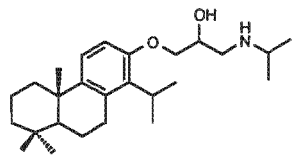

Appendix 3 - NMR and IR Spectra

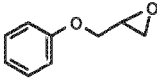
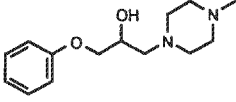
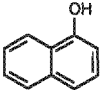
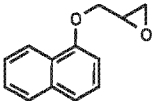
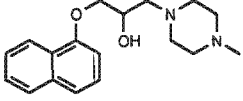
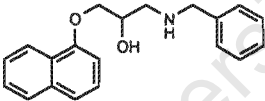
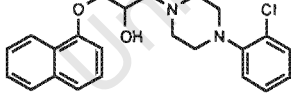
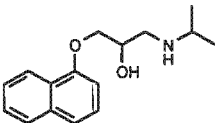
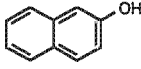
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<u>Figure A. 3.4.1.</u> ^1H NMR of Compound 6 , 300MHz, CDCl_3	150

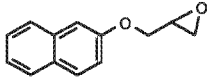
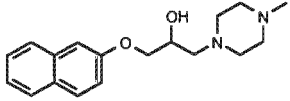
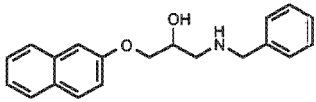
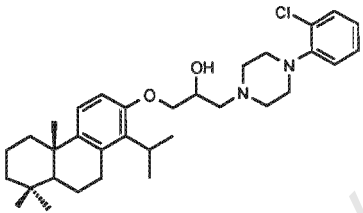
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Compound	Structure	Molecular Formula	Molecular Weight (g/mol)	c log P
3		C ₂₀ H ₃₀ O	286.4516	6.46
4		C ₂₃ H ₃₄ O ₂	342.51486	6.3
5		C ₂₈ H ₄₆ N ₂ O ₂	442.67712	5.94
6		C ₃₁ H ₃₉ NO ₄	489.6457	6.93
7		C ₃₀ H ₄₃ NO ₂	449.66796	7.54
8		C ₂₄ H ₃₈ O ₃	374.55672	6.03
9		C ₂₆ H ₄₃ NO ₂	401.62516	6.46
10		C ₆ H ₆ O	94.11304	1.48

Compound	Structure	Molecular Formula	Molecular Weight (g/mol)	c log P
11		$C_9H_{10}O_2$	150.1745	1.56
12		$C_{14}H_{22}N_2O_2$	250.33676	1.13
13		$C_{10}H_8O$	144.17292	2.60
14		$C_{13}H_{12}O_2$	200.23708	2.68
15		$C_{18}H_{24}N_2O_2$	300.39544	2.12
16		$C_{20}H_{21}NO_2$	307.38628	3.72
17		$C_{23}H_{25}ClN_2O_2$	396.90958	4.76
18		$C_{16}H_{21}NO_2$	259.34348	2.65
19		$C_{10}H_8O$	144.17292	2.60

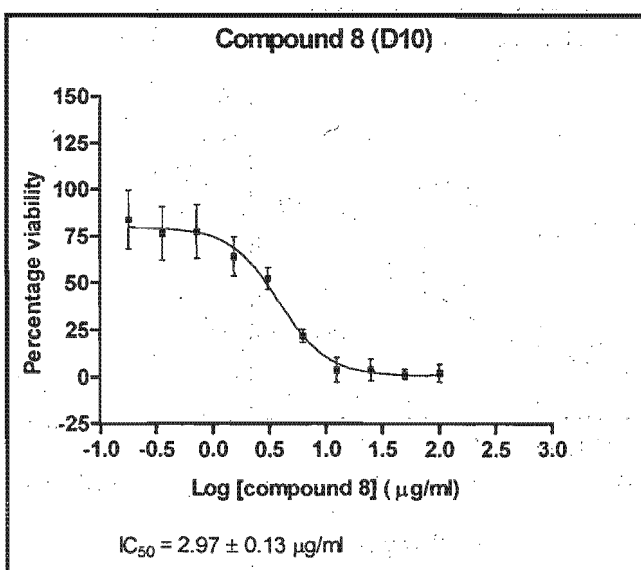
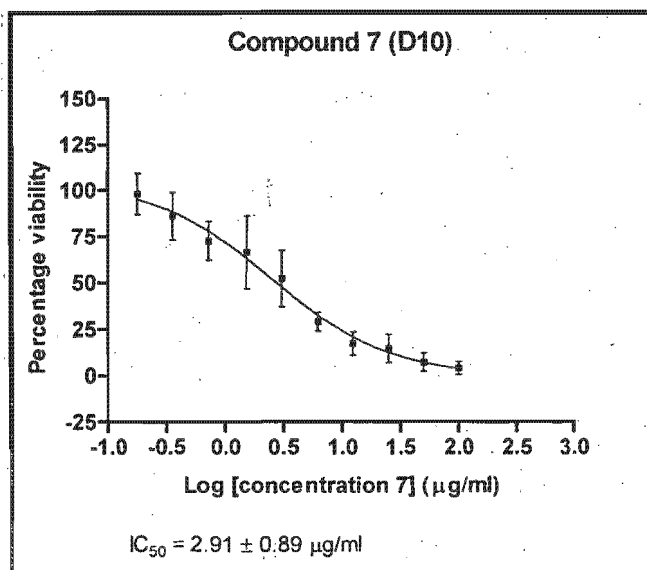
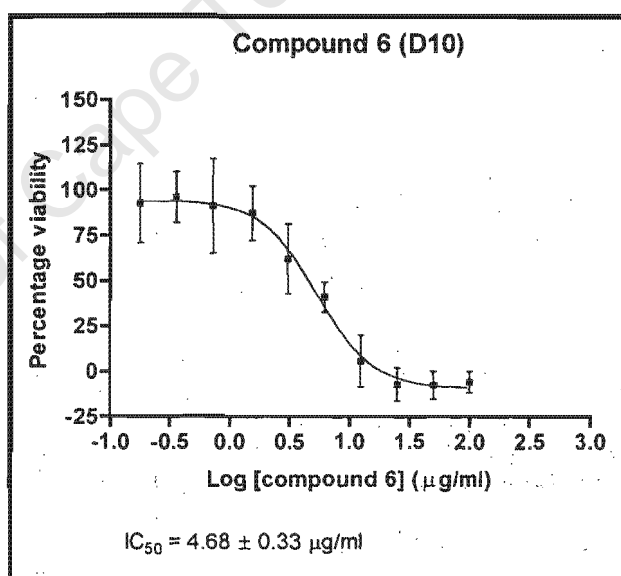
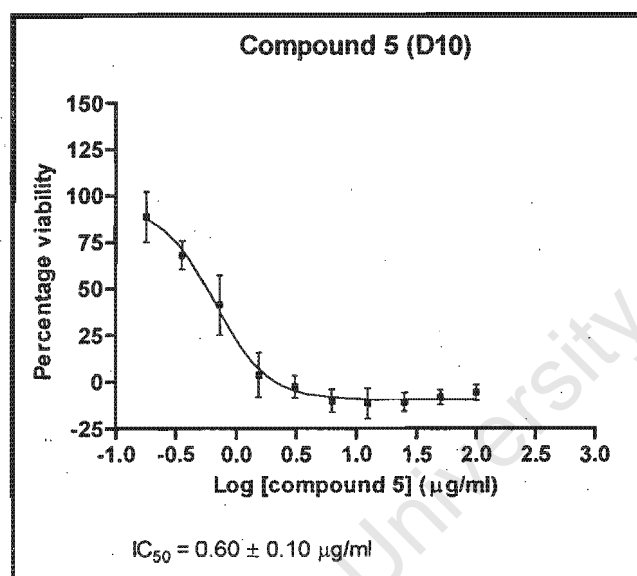
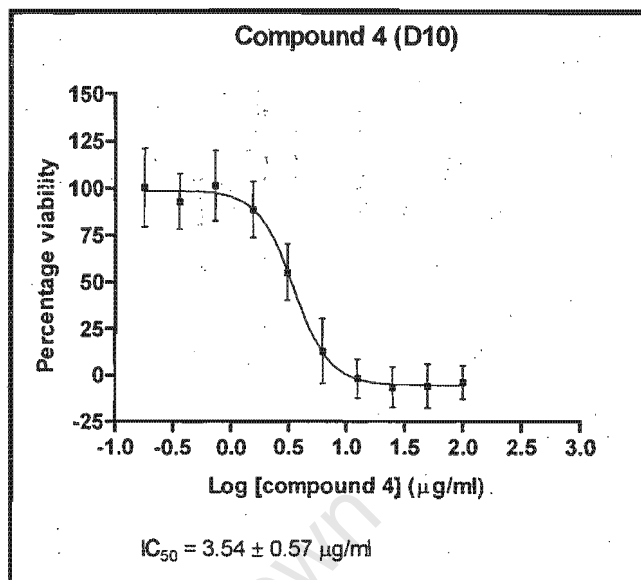
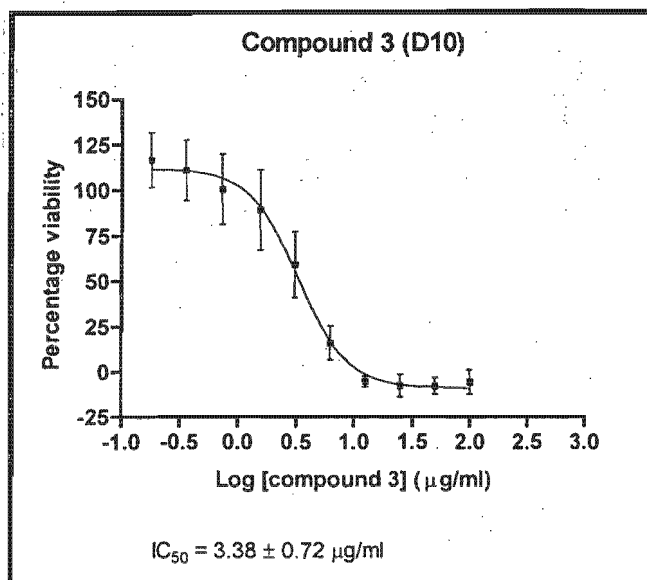
Compound	Structure	Molecular Formula	Molecular Weight (g/mol)	c log P
20		C ₁₃ H ₁₂ O ₂	200.23708	2.68
21		C ₁₈ H ₂₄ N ₂ O ₂	300.39544	2.12
22		C ₂₀ H ₂₁ NO ₂	307.38628	3.72
4*		C ₂₈ H ₄₆ N ₂ O ₉	539.19126	8.57

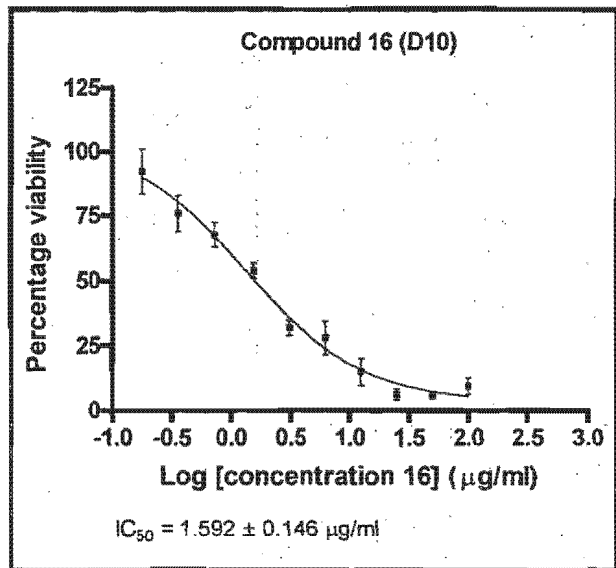
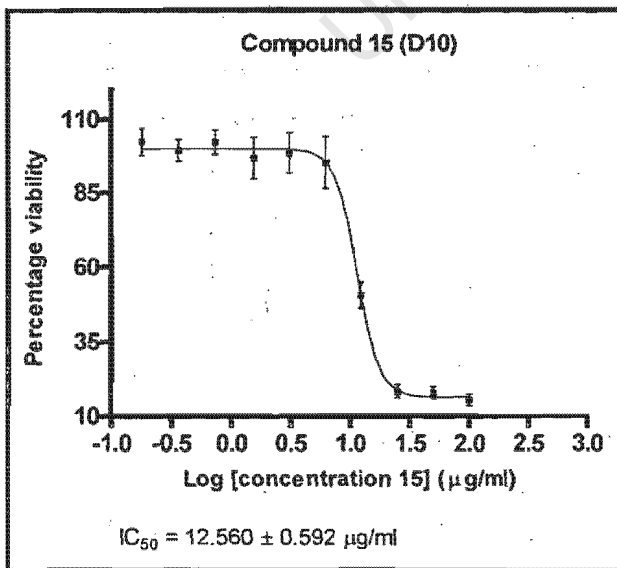
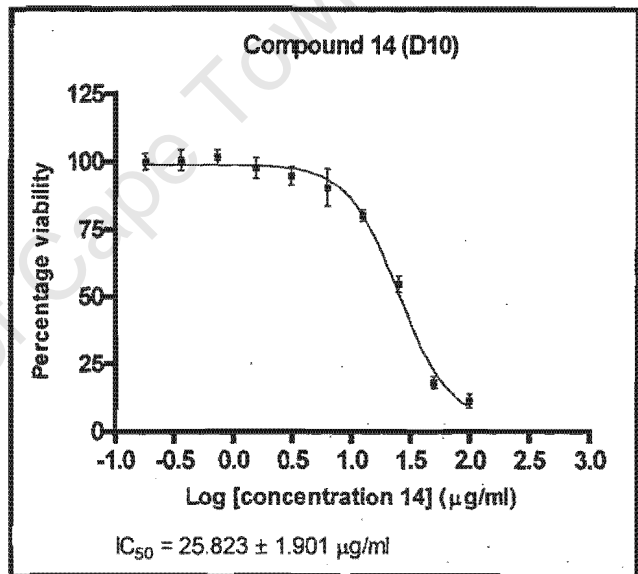
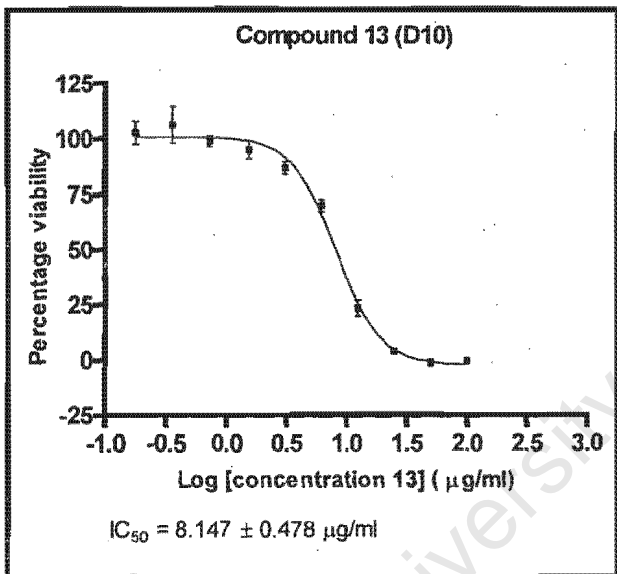
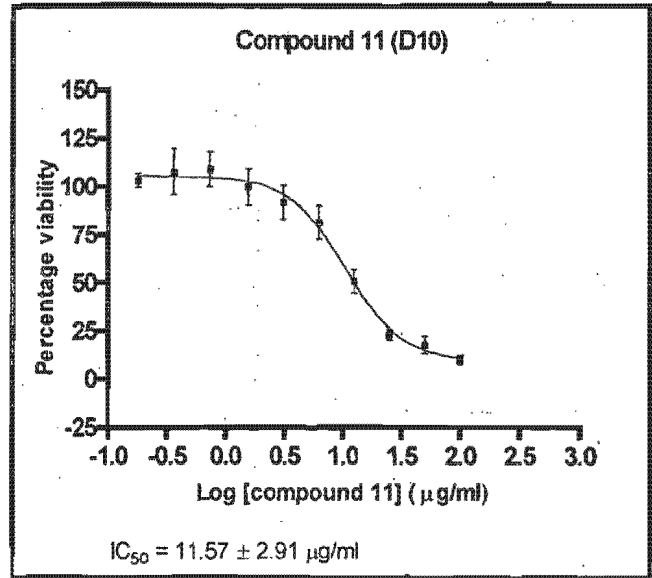
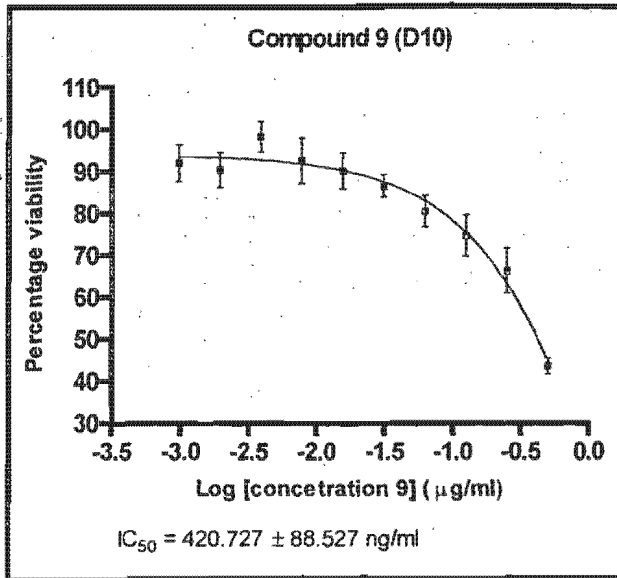
Appendix 2

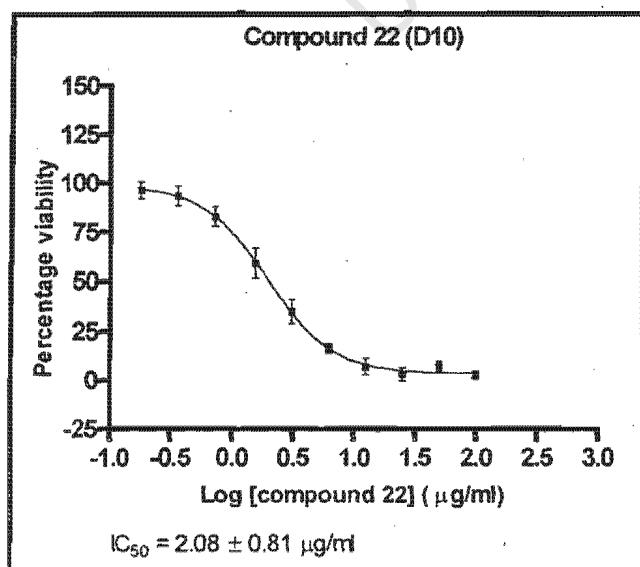
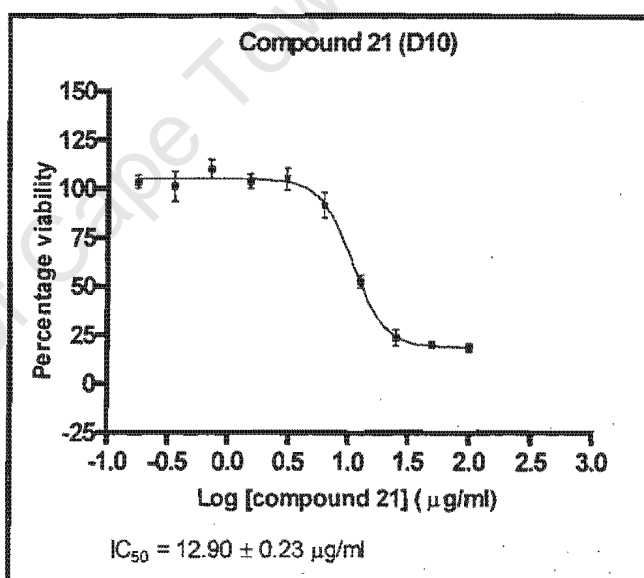
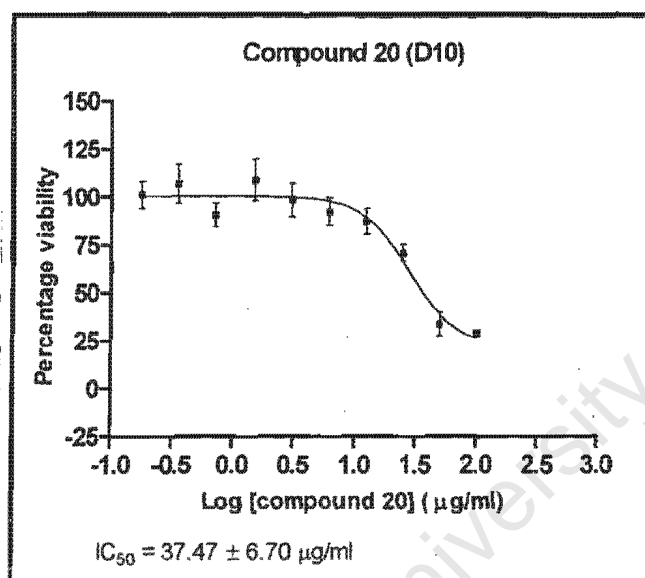
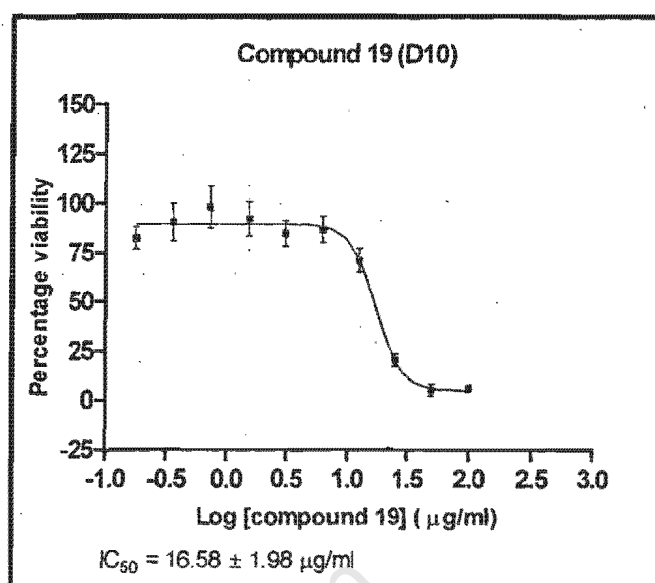
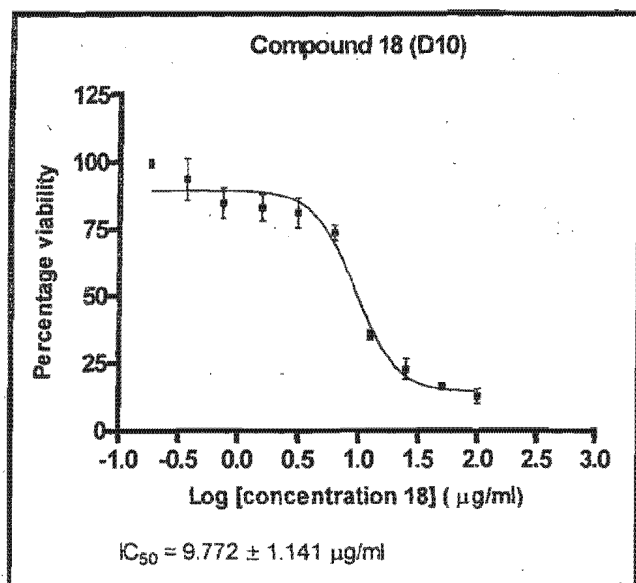
Dose Response Curves

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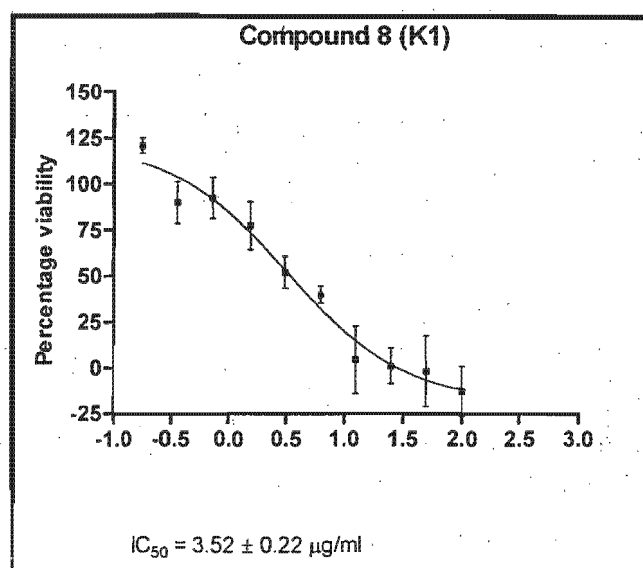
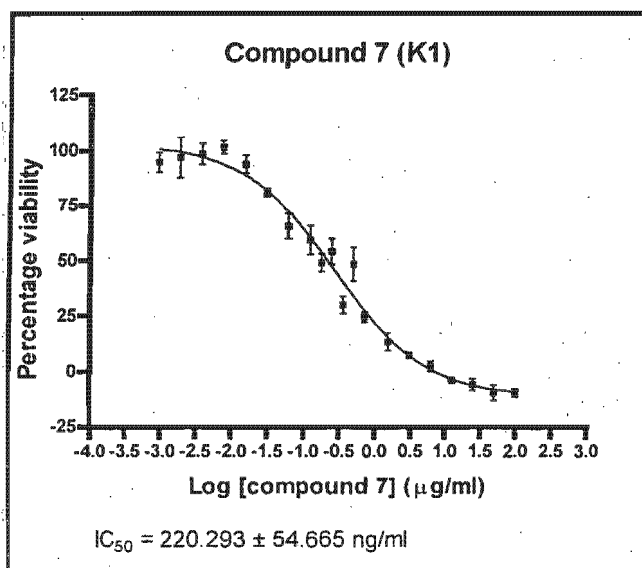
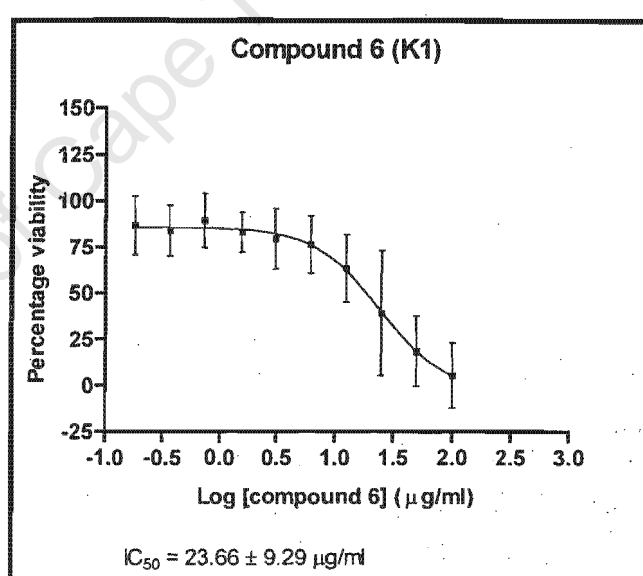
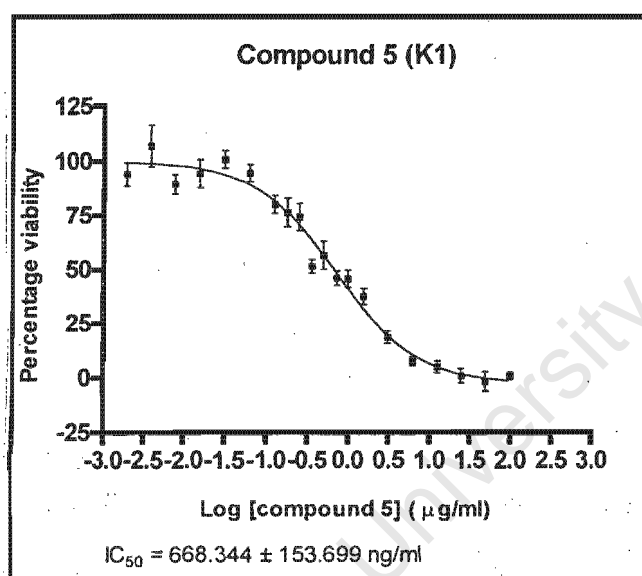
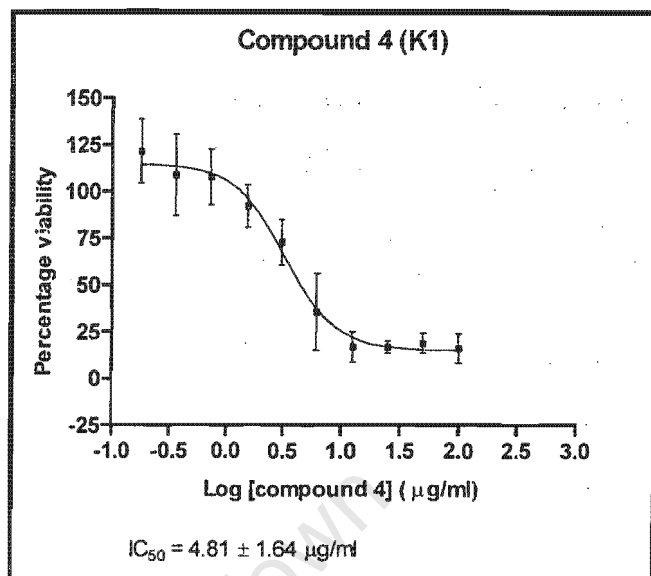
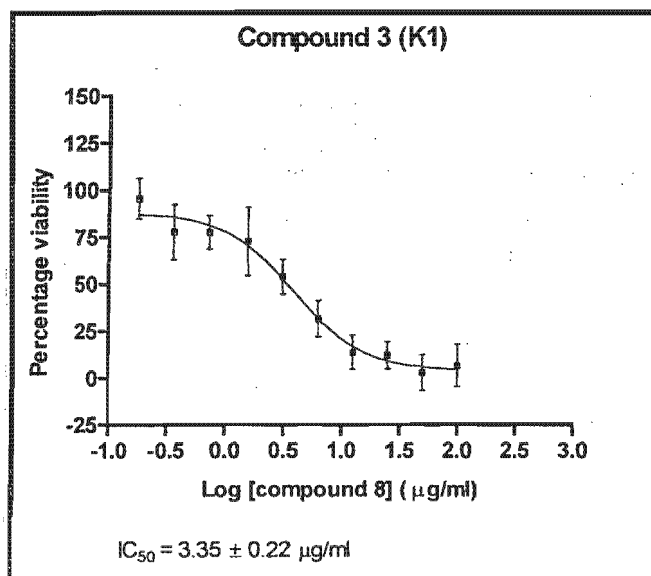
Dose response curves of compounds 3-9, 11, 13-16 and 18-22 on a CQ sensitive strain (D10)

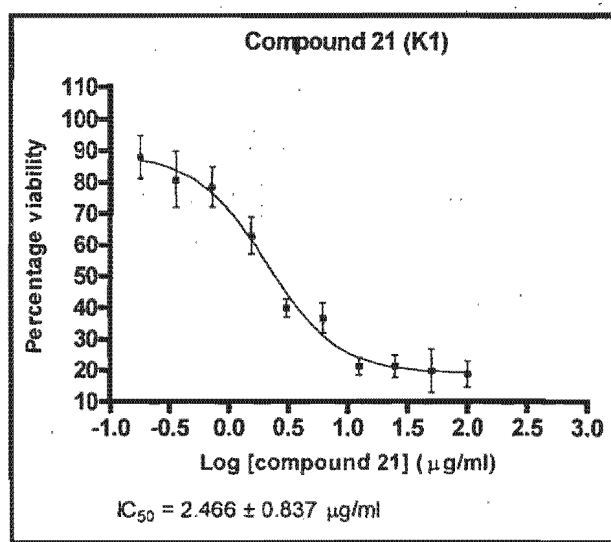
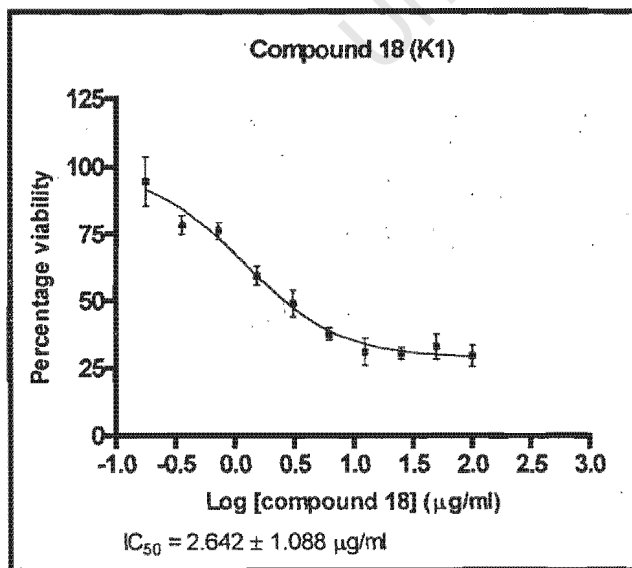
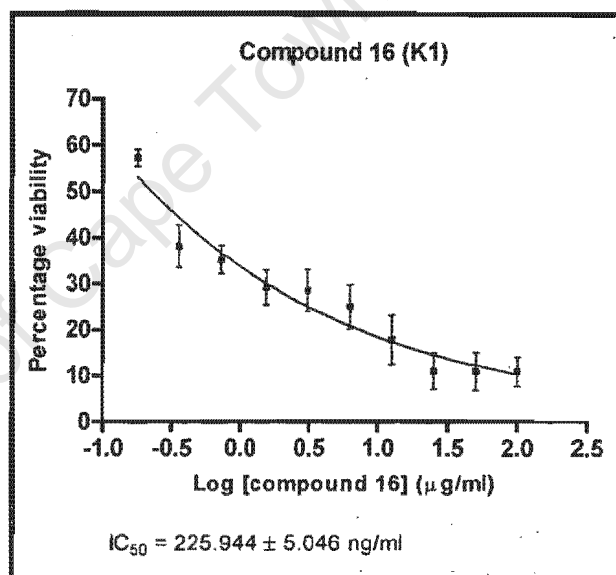
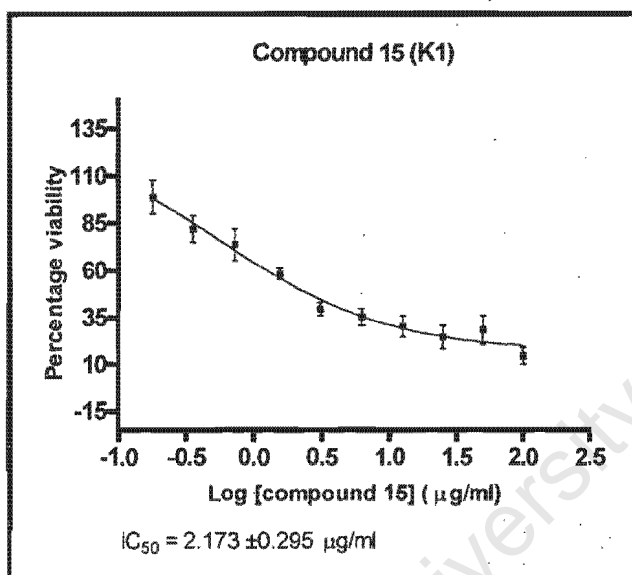
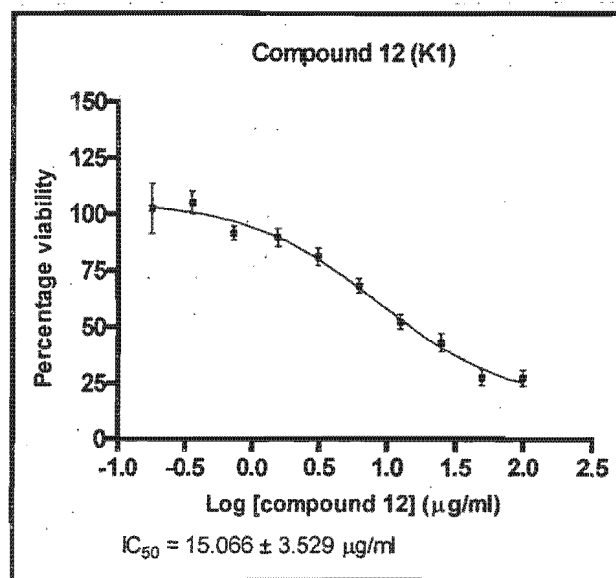
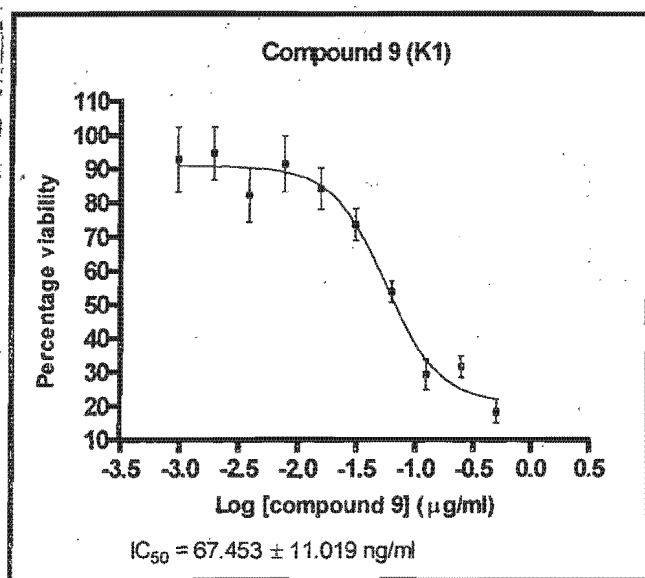


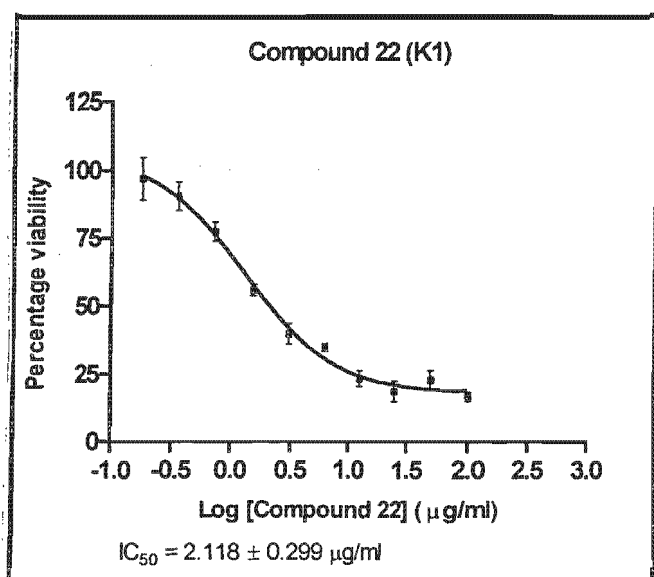




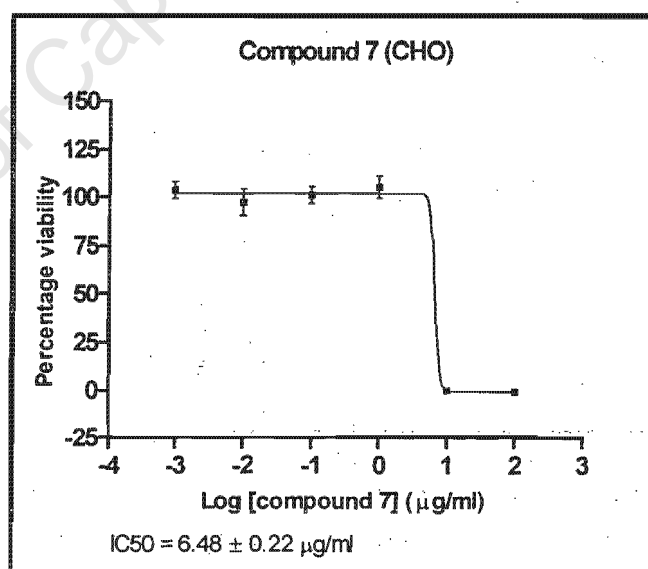
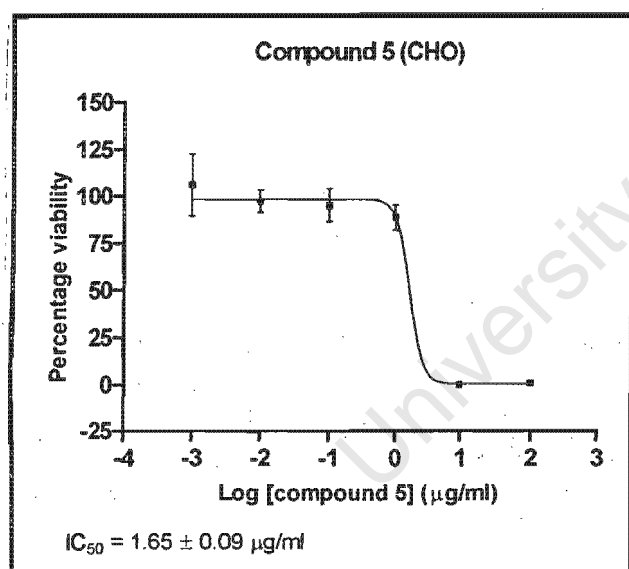
Dose response curves of compounds 3-9, 12, 15, 16, 18, 21 and 22 on a CQ resistant strain (K1)

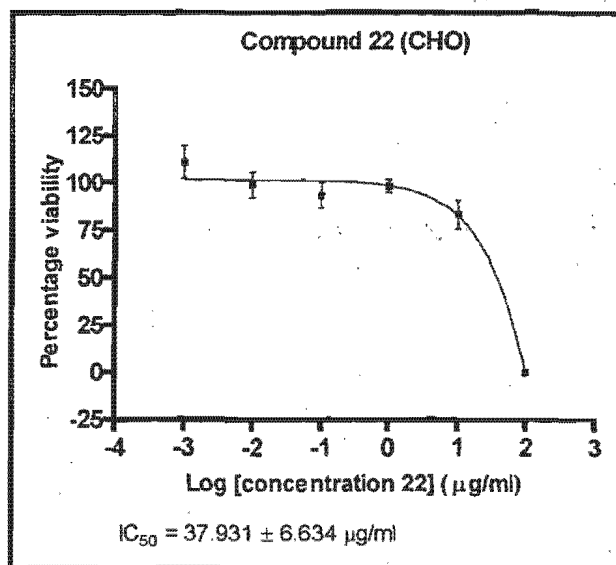
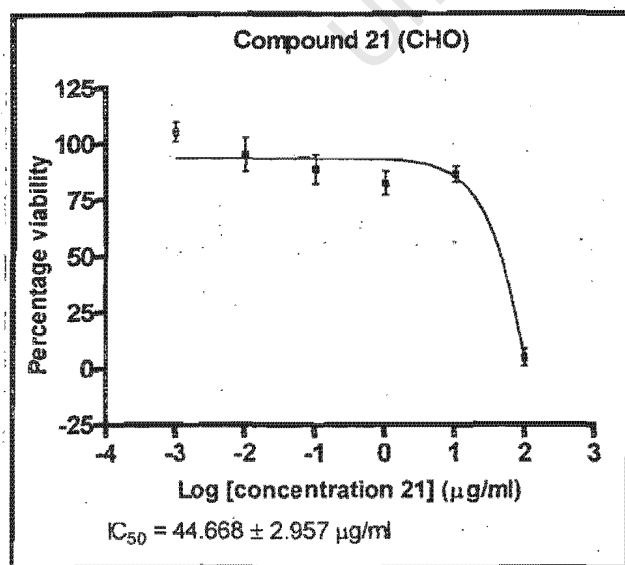
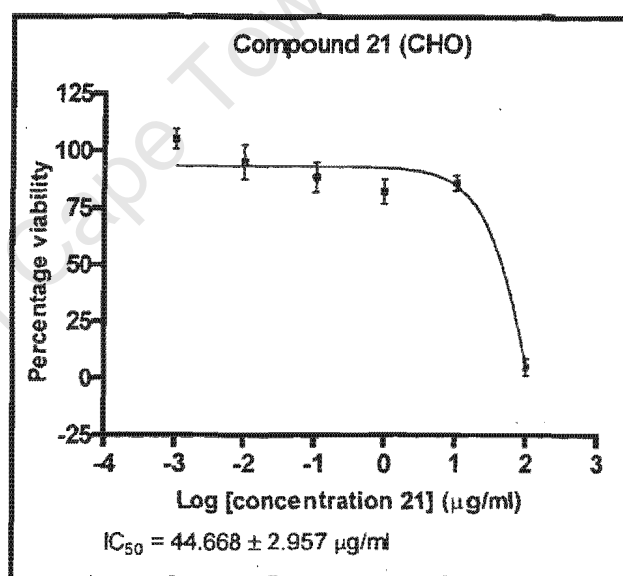
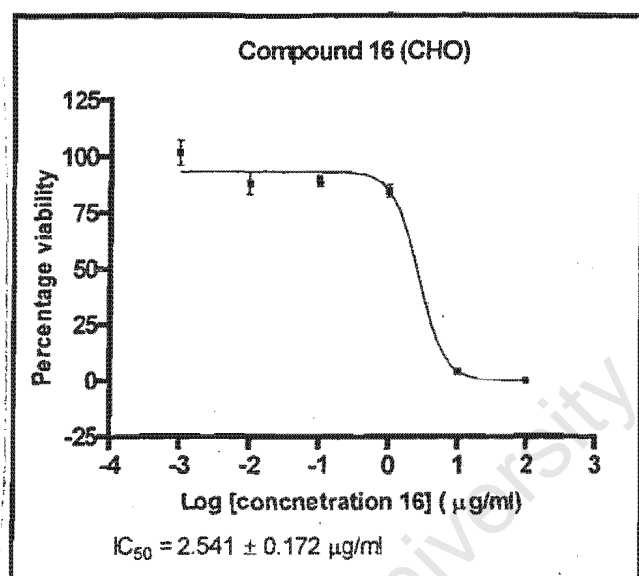
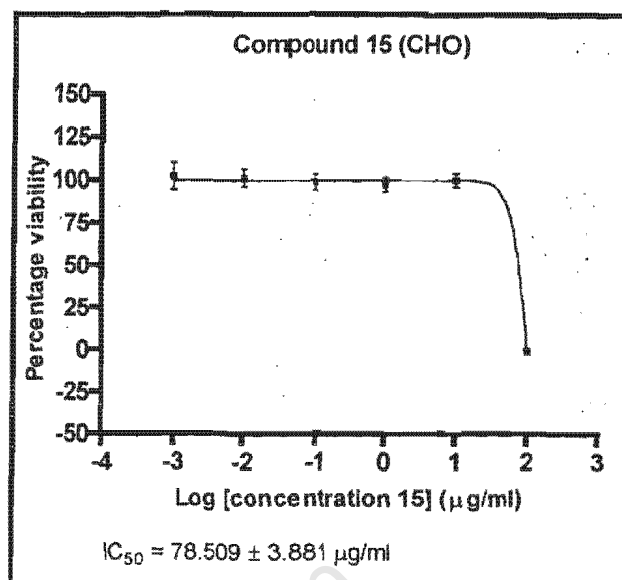
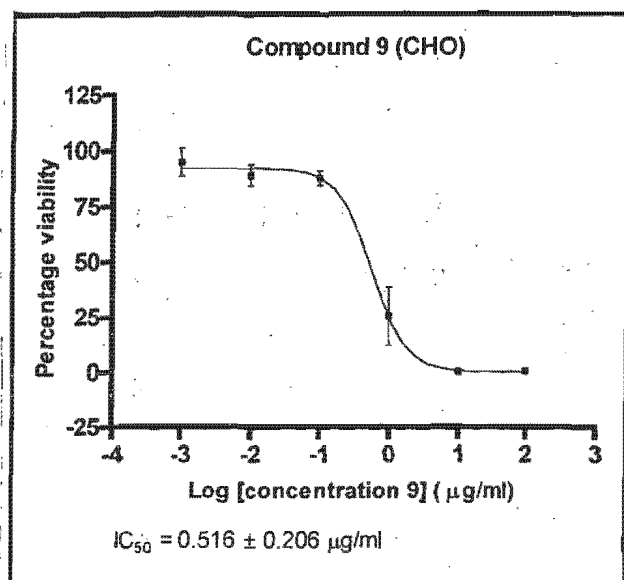






Dose response curves of compounds 5, 7, 9, 15, 16, 21 and 22 on a CHO cells





Appendix 3

NMR and IR Spectra

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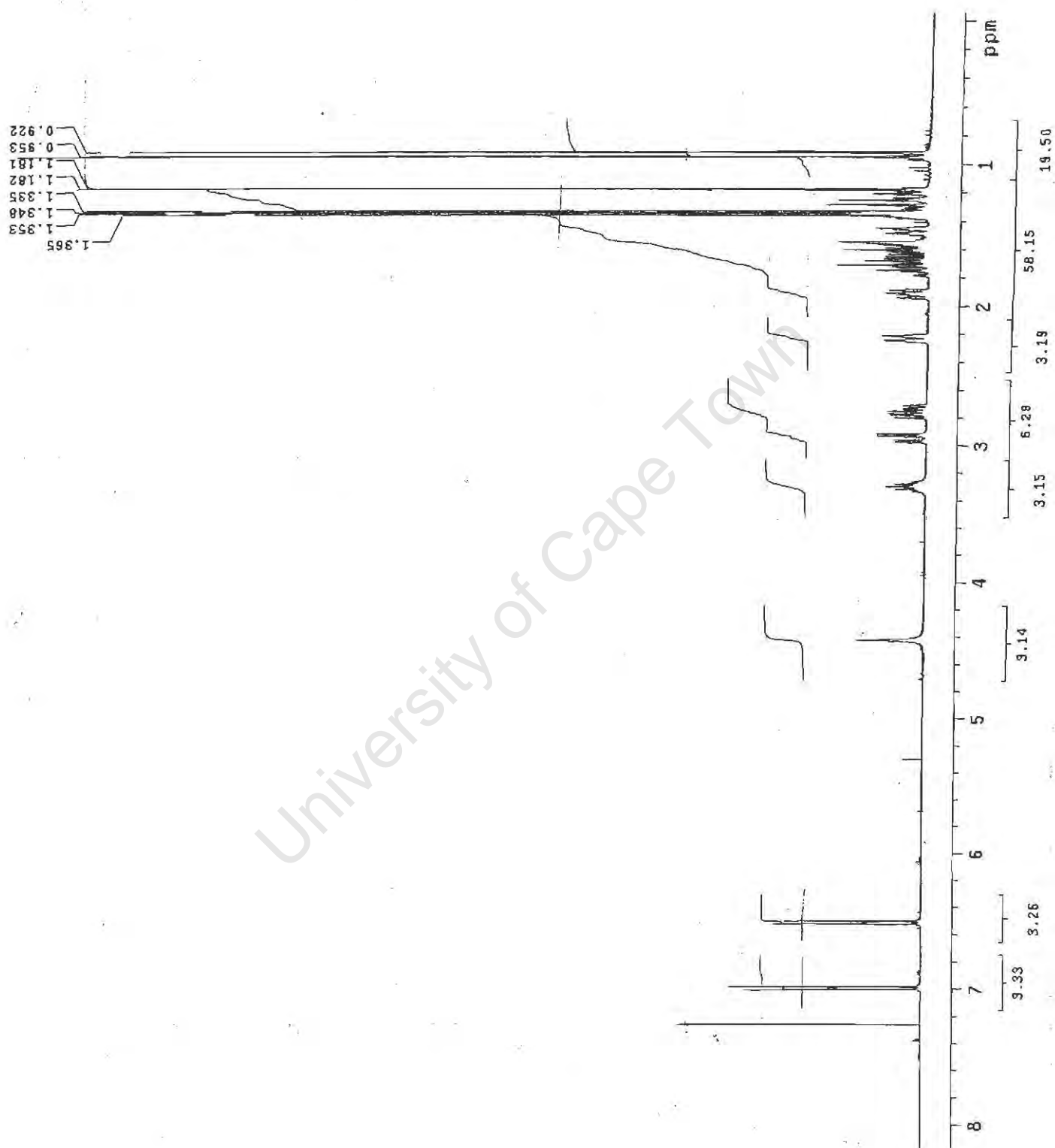
Figure A.3.1.1.: ^1H NMR of Totarol (3)

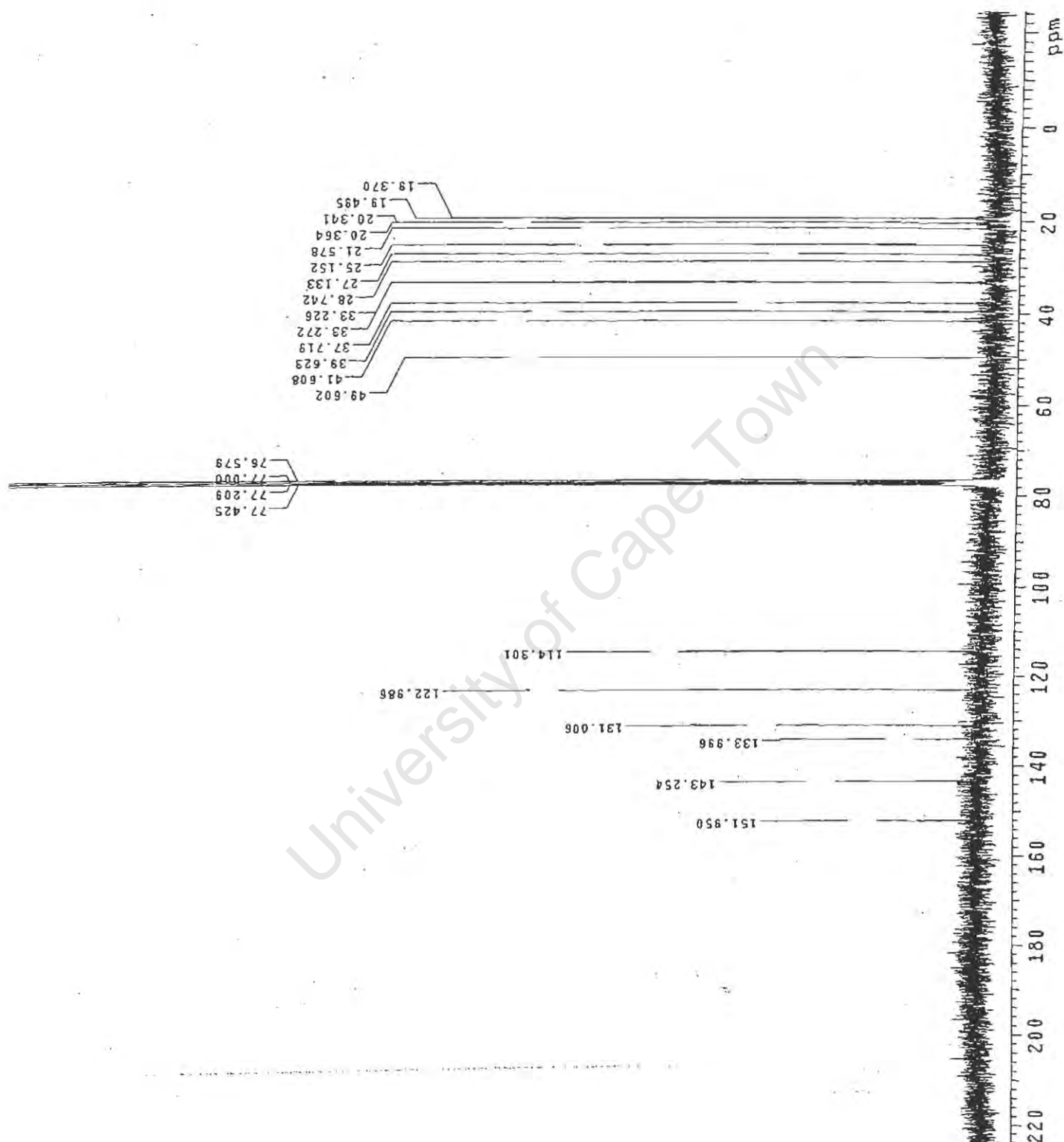
Figure A.3.1.2.: ^{13}C NMR of Tatarol (3)

Figure A.3.1.3.: COSY of Totarol (3)

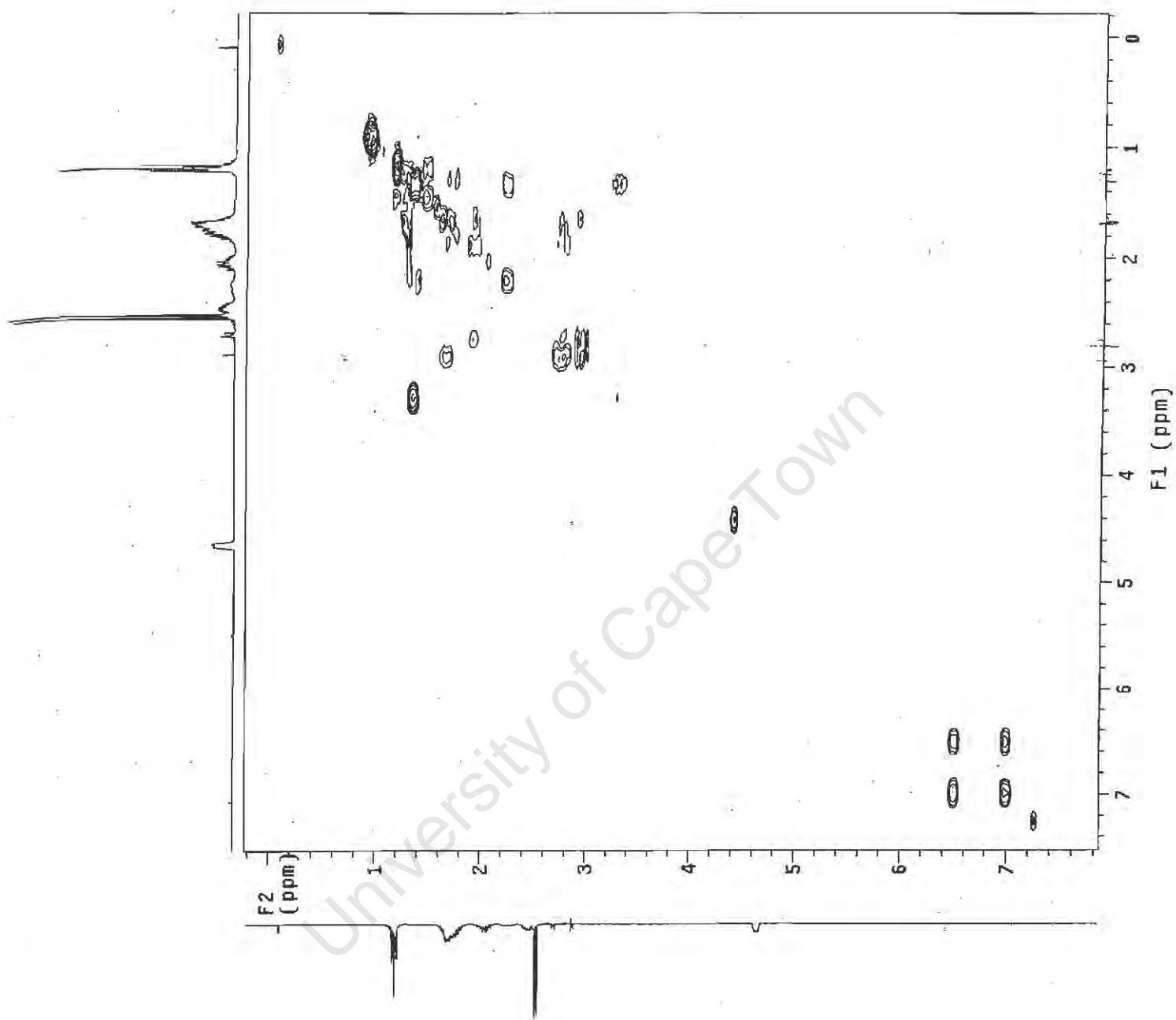


Figure A.3.1.4.: HMBC of Totarol (3)

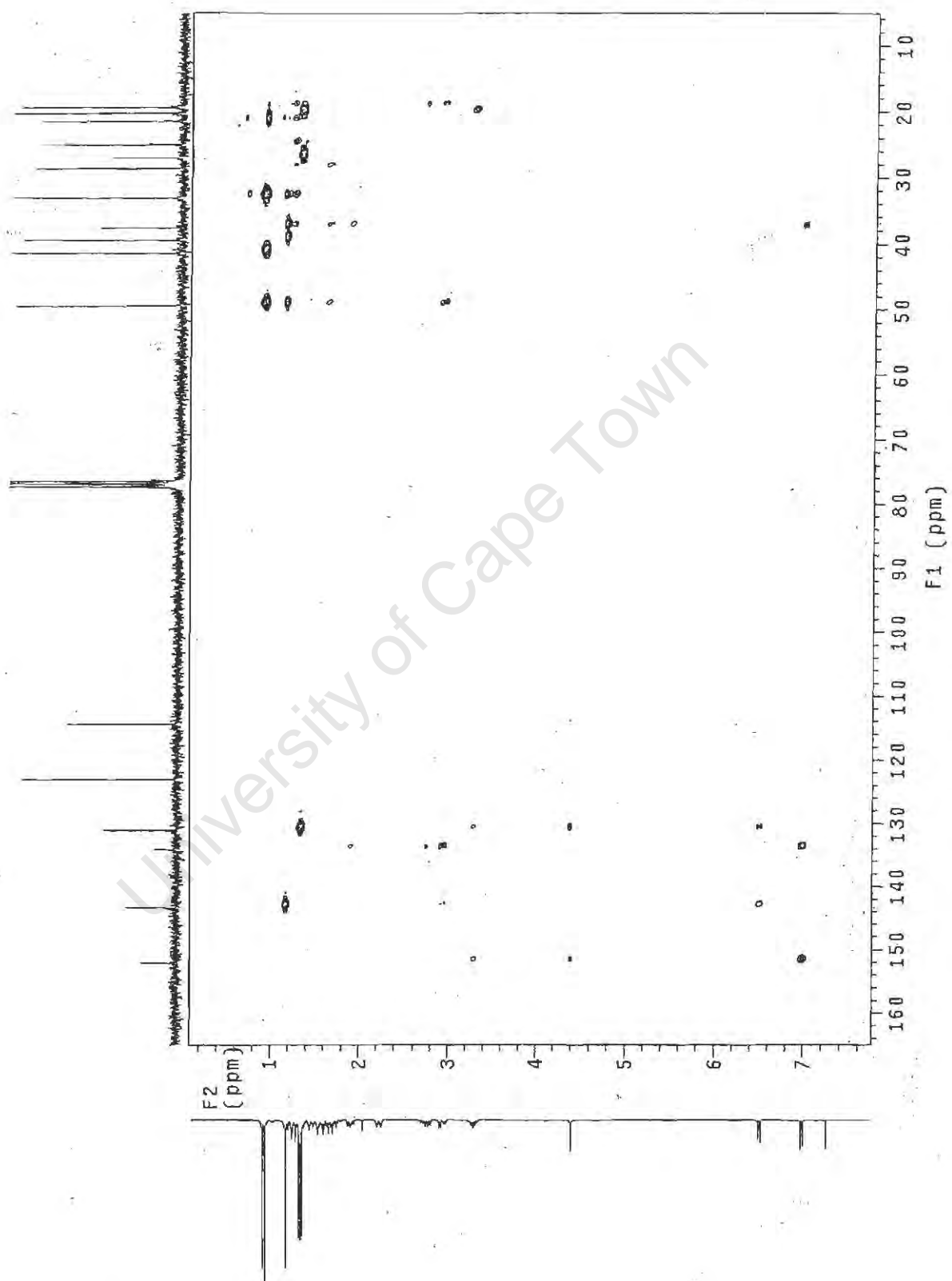


Figure A.3.1.5.: HSQC of Totarol (3)

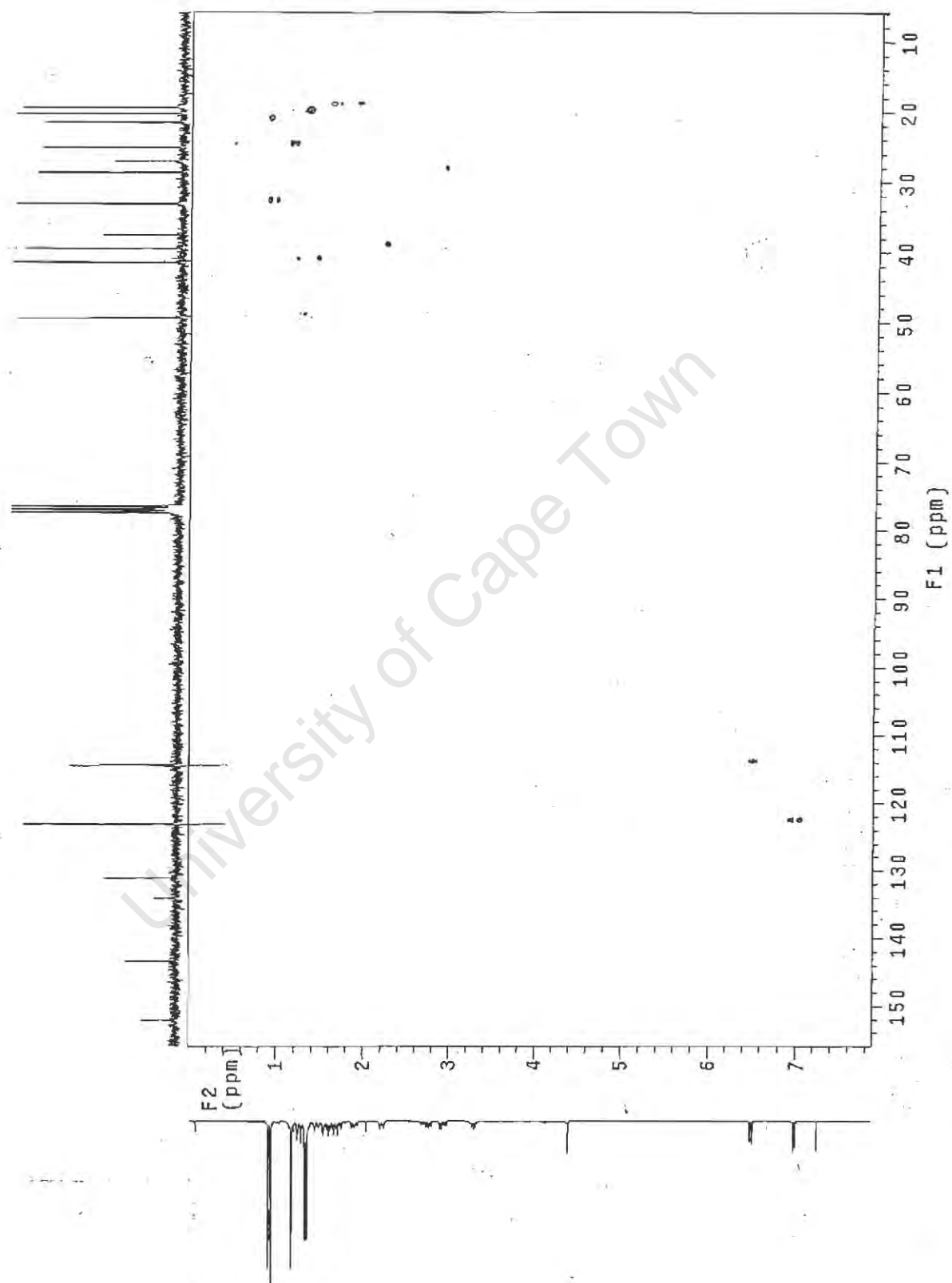


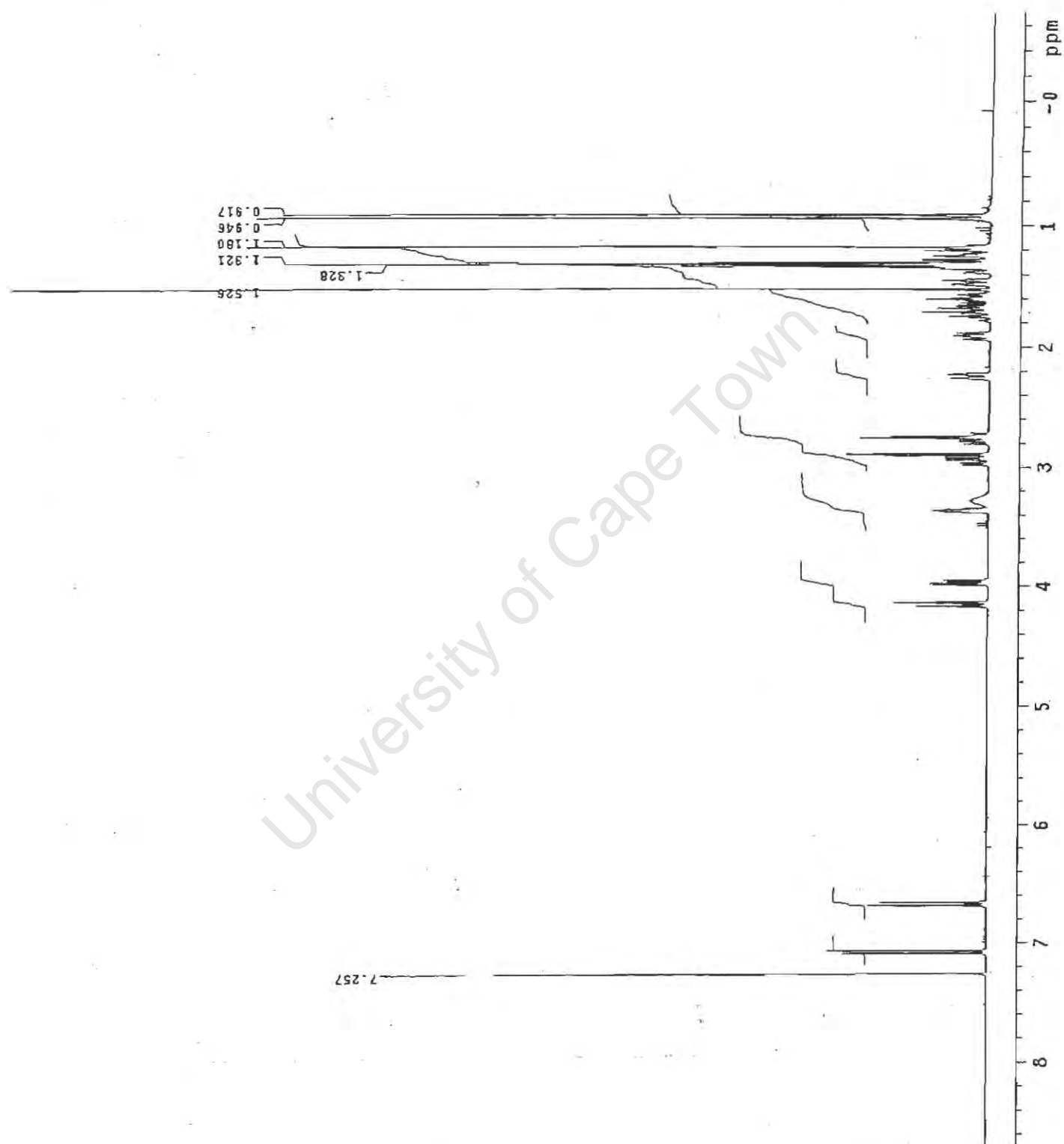
Figure A.3.2.1.: ^1H NMR of Compound **4**

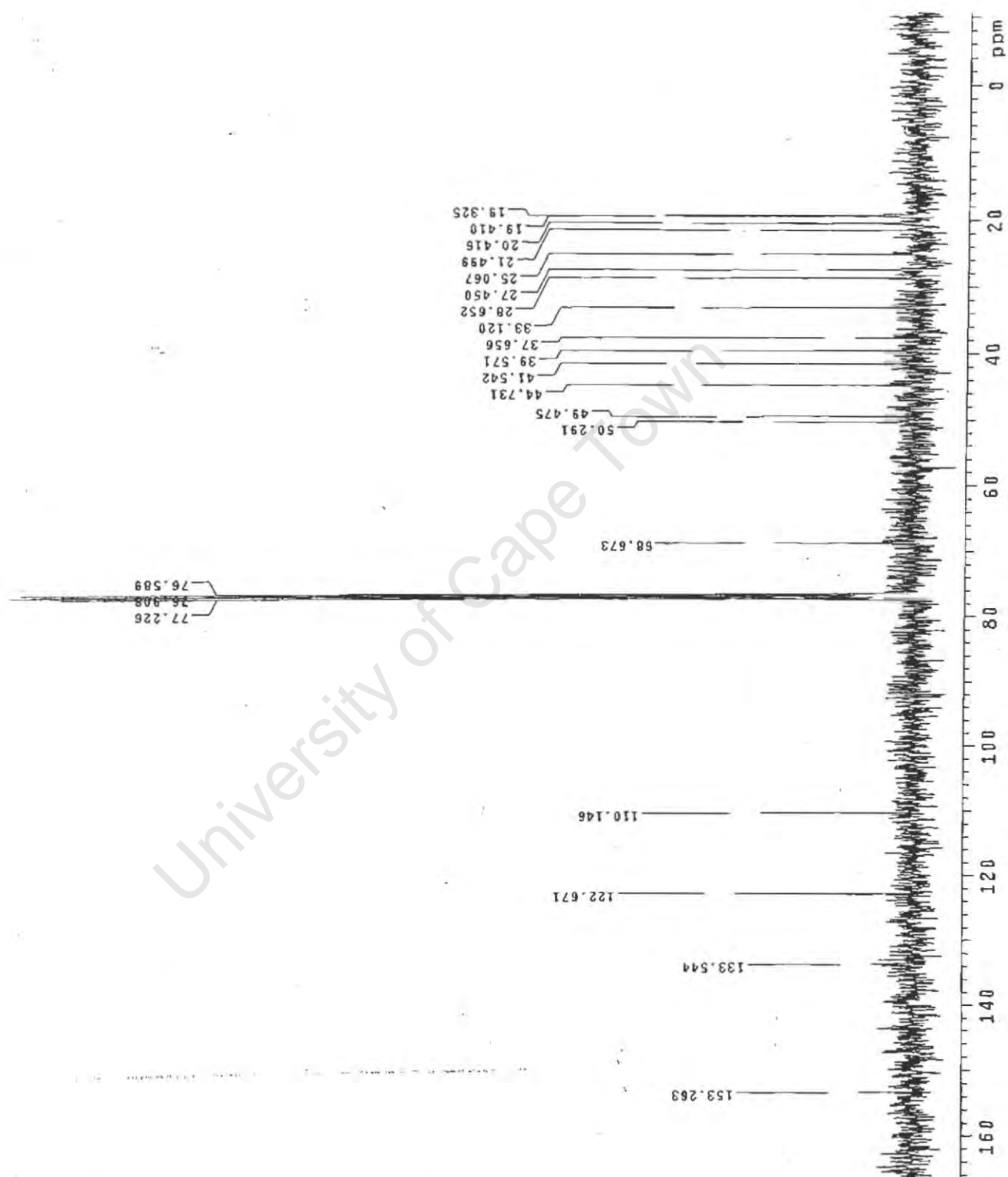
Figure A.3.2.2.: ^{13}C NMR of Compound 4

Figure A.3.2.3.: COSY of Compound 4

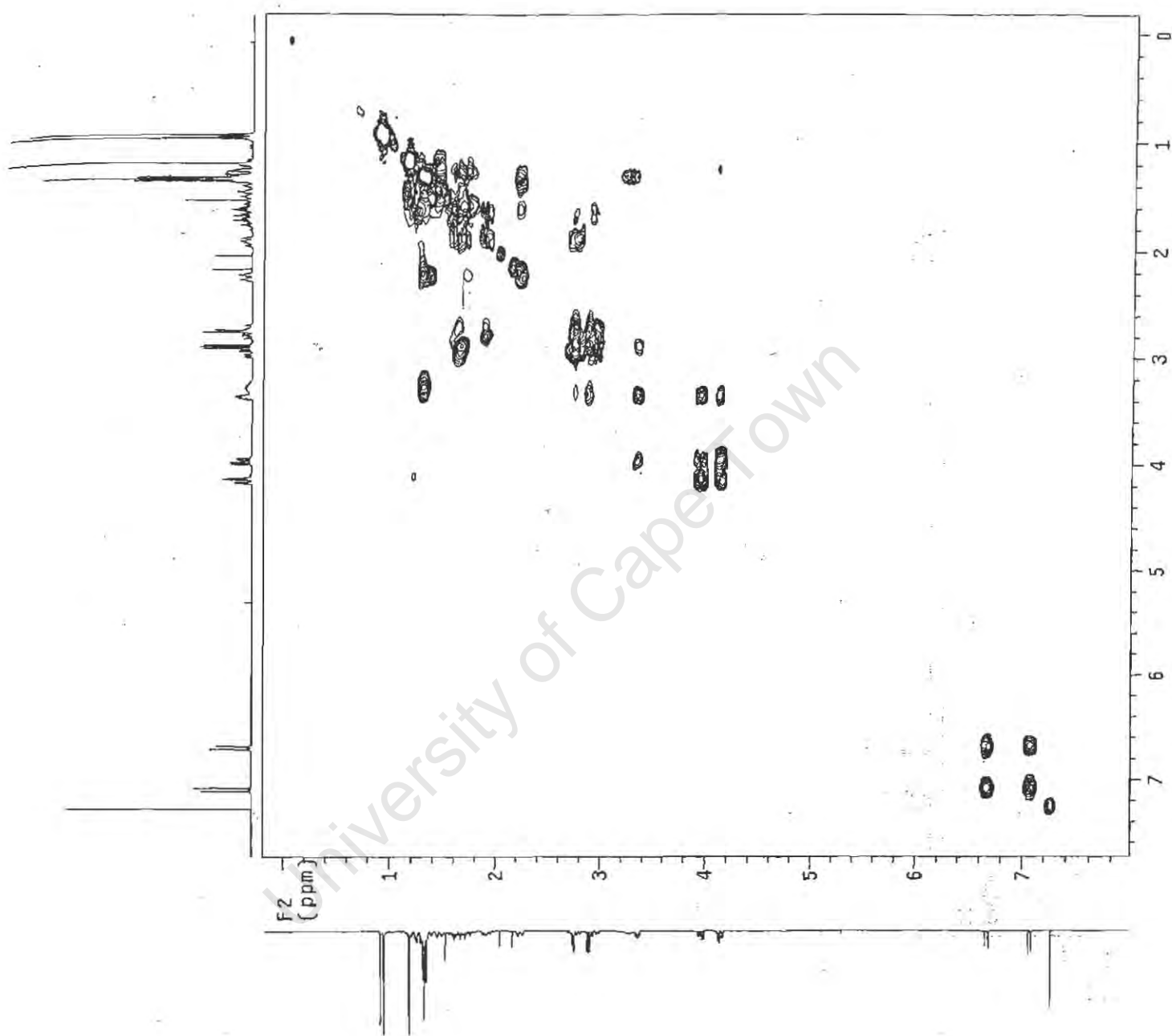


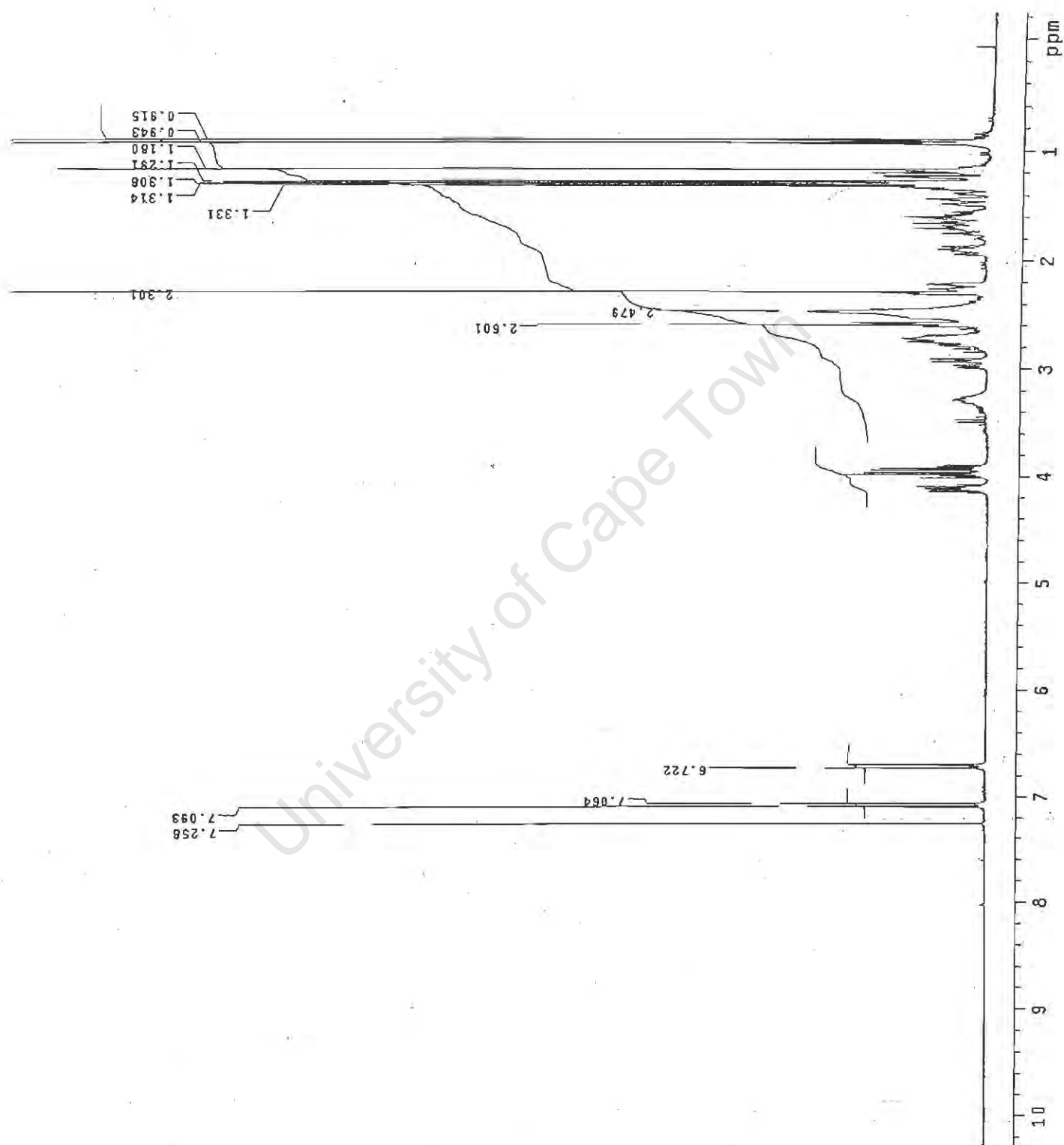
Figure A.3.3.1.: ^1H NMR of Compound 5

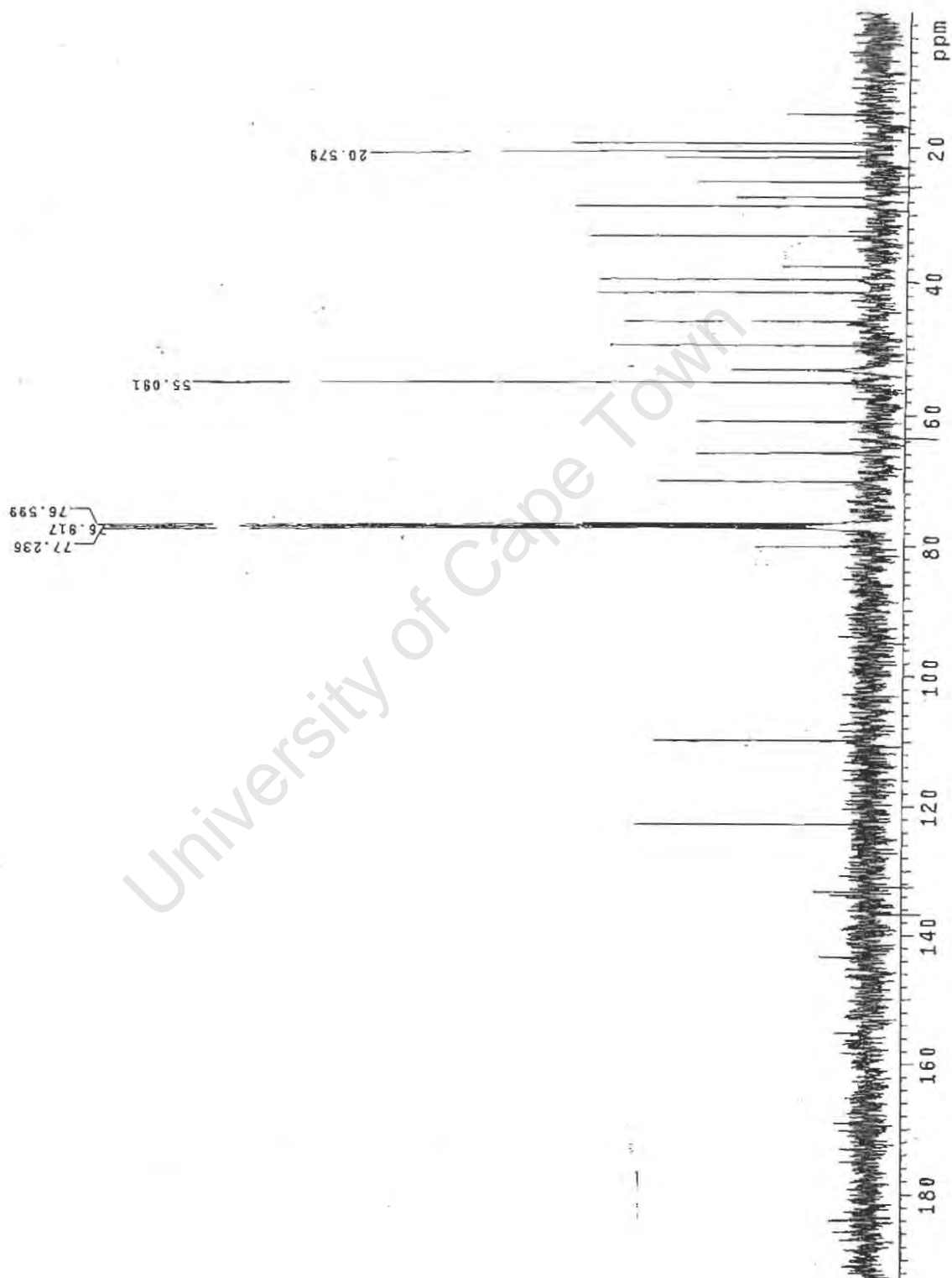
Figure A.3.3.2.: ^{13}C NMR of Compound 5

Figure A.3.3.3.: COSY of Compound 5

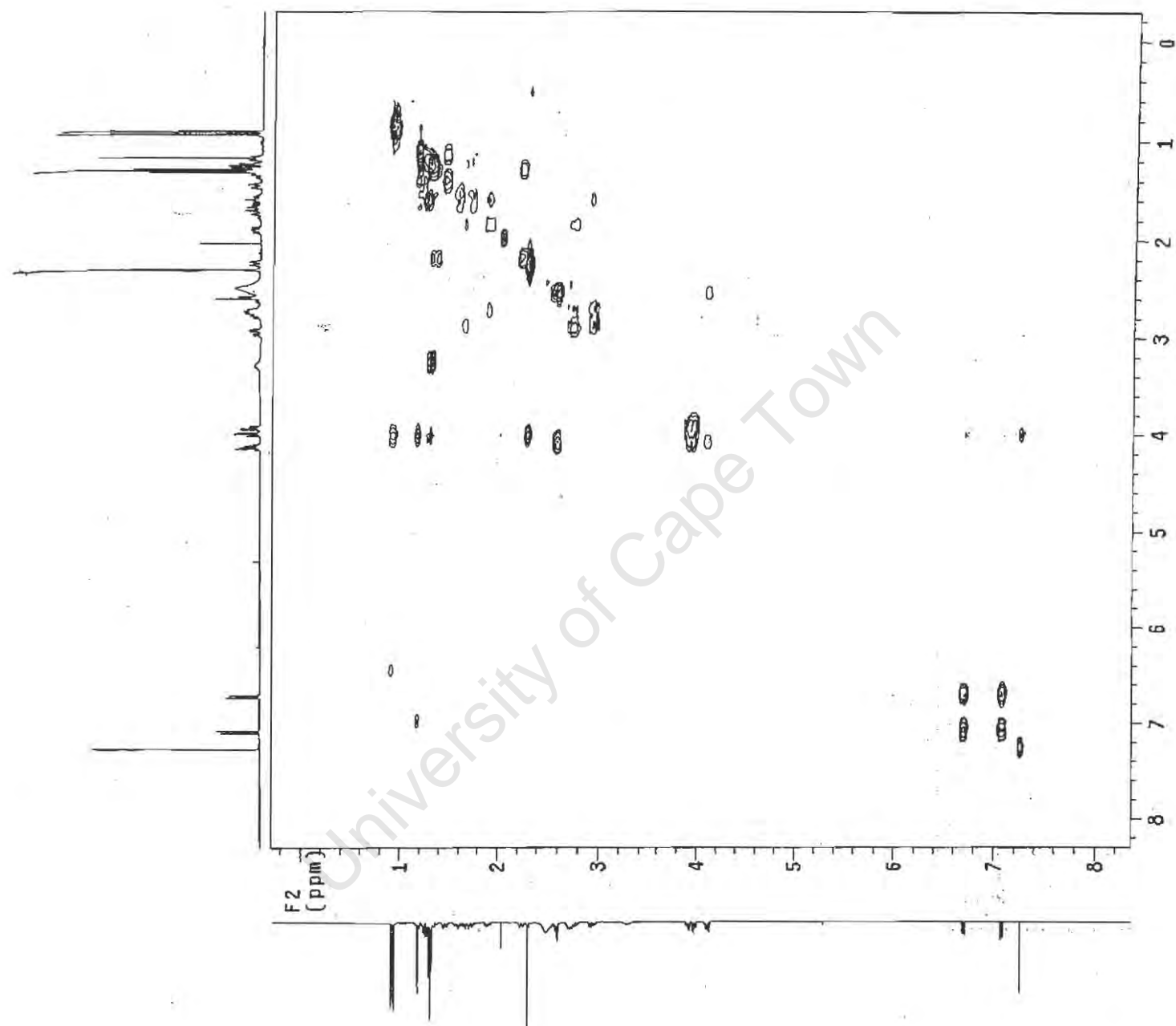
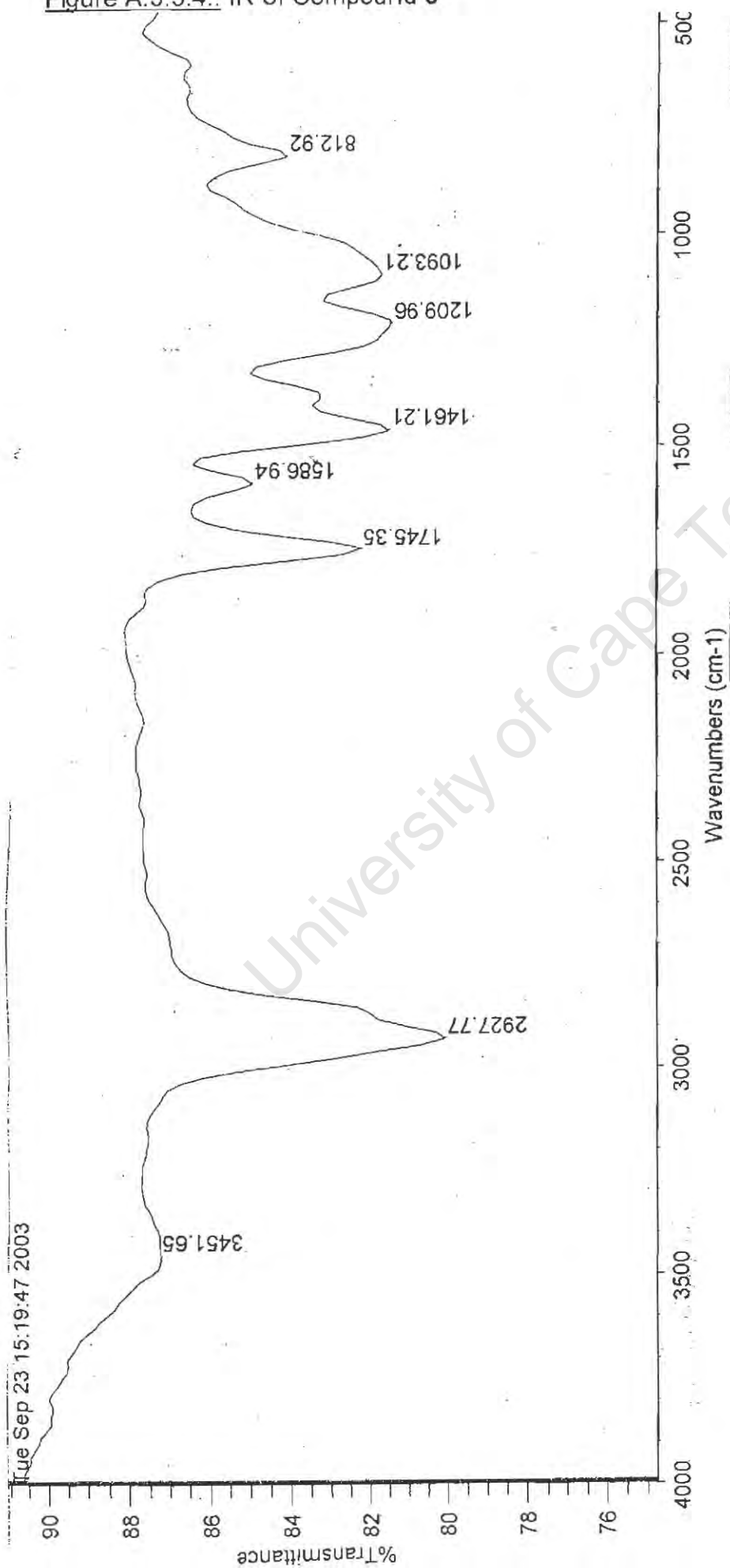


Figure A.3.3.4.: IR of Compound 5



Tue Sep 23 15:19:47 2003

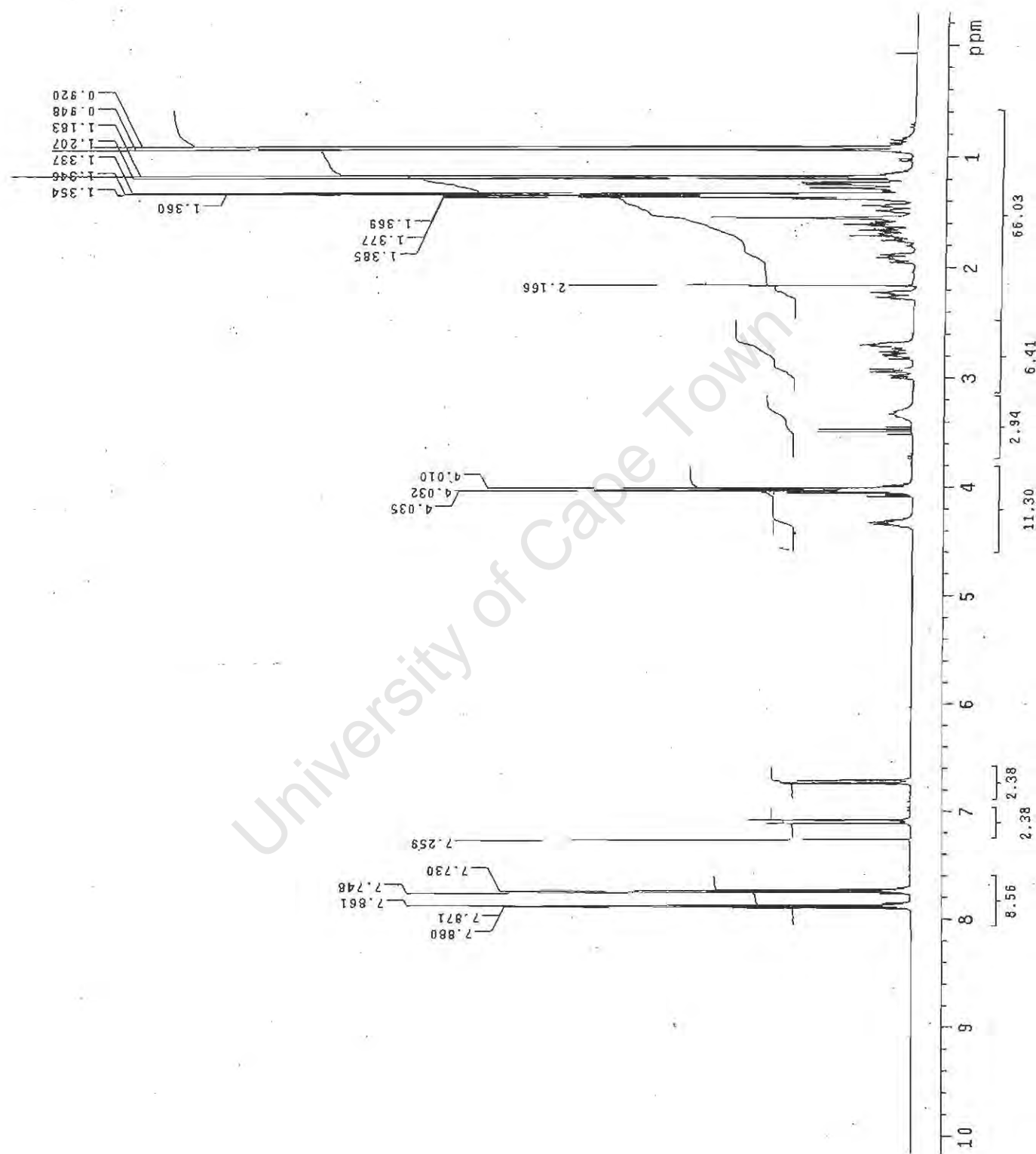
Figure A.3.4.1.: ^1H NMR of Compound 6

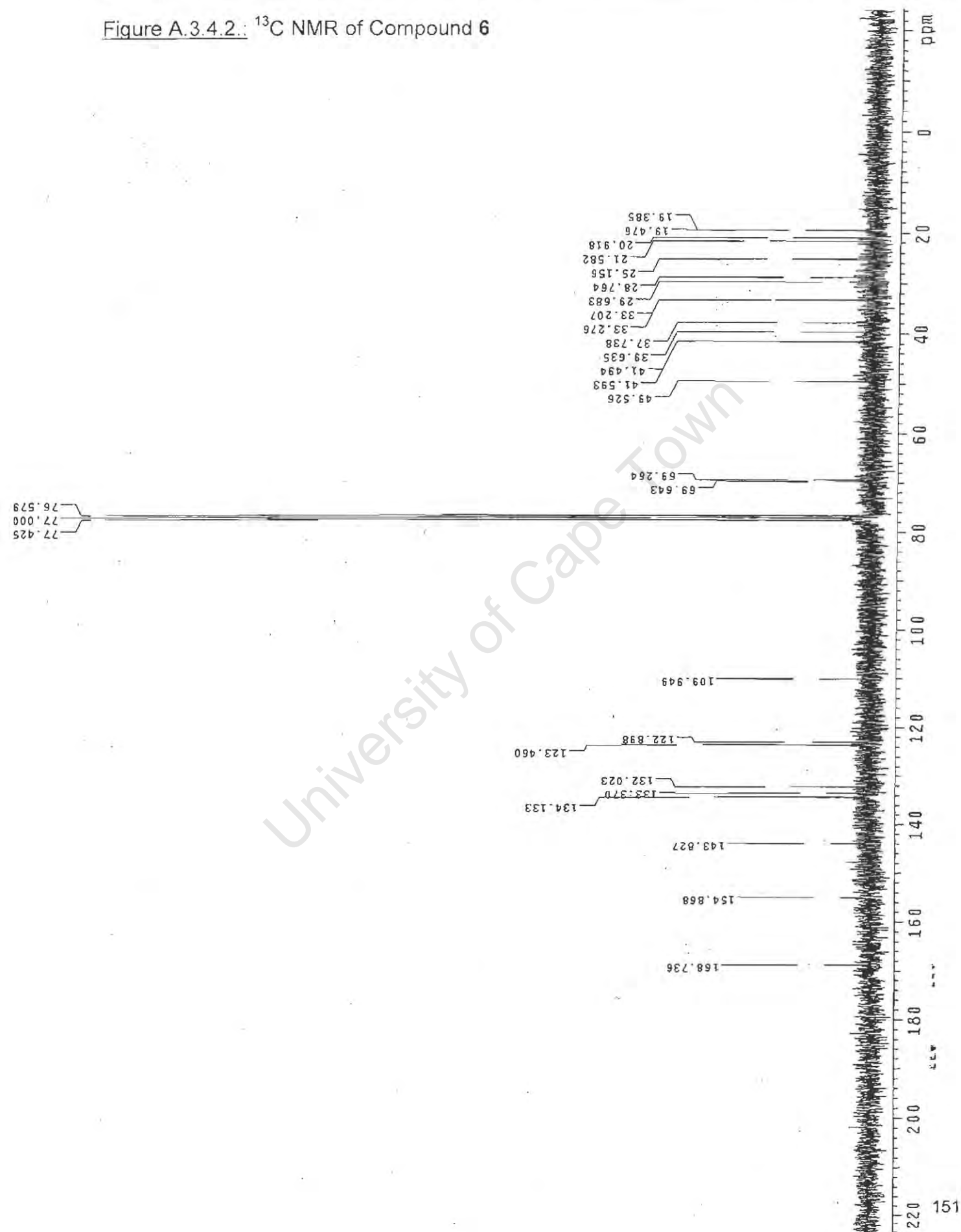
Figure A.3.4.2.: ^{13}C NMR of Compound 6

Figure A.3.4.3.: IR of Compound 6

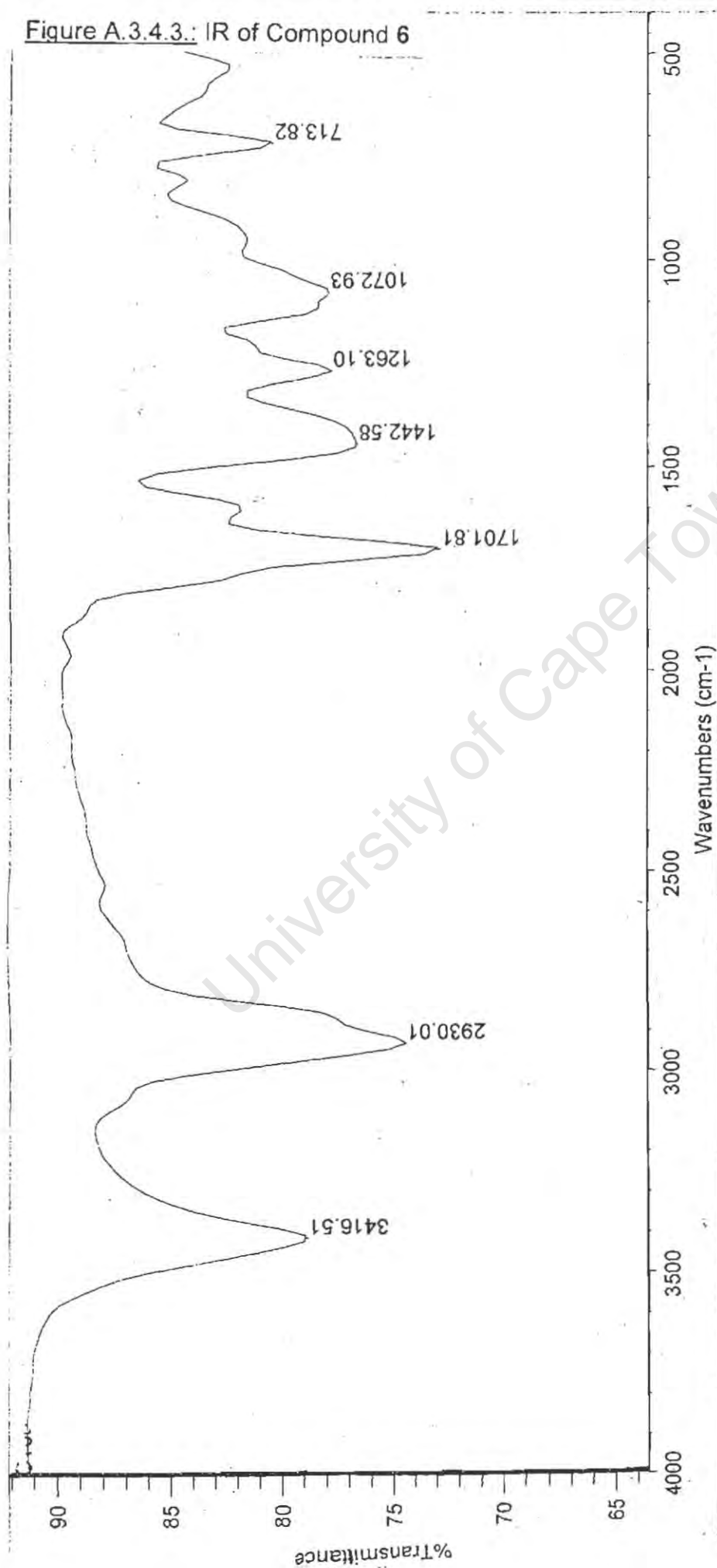


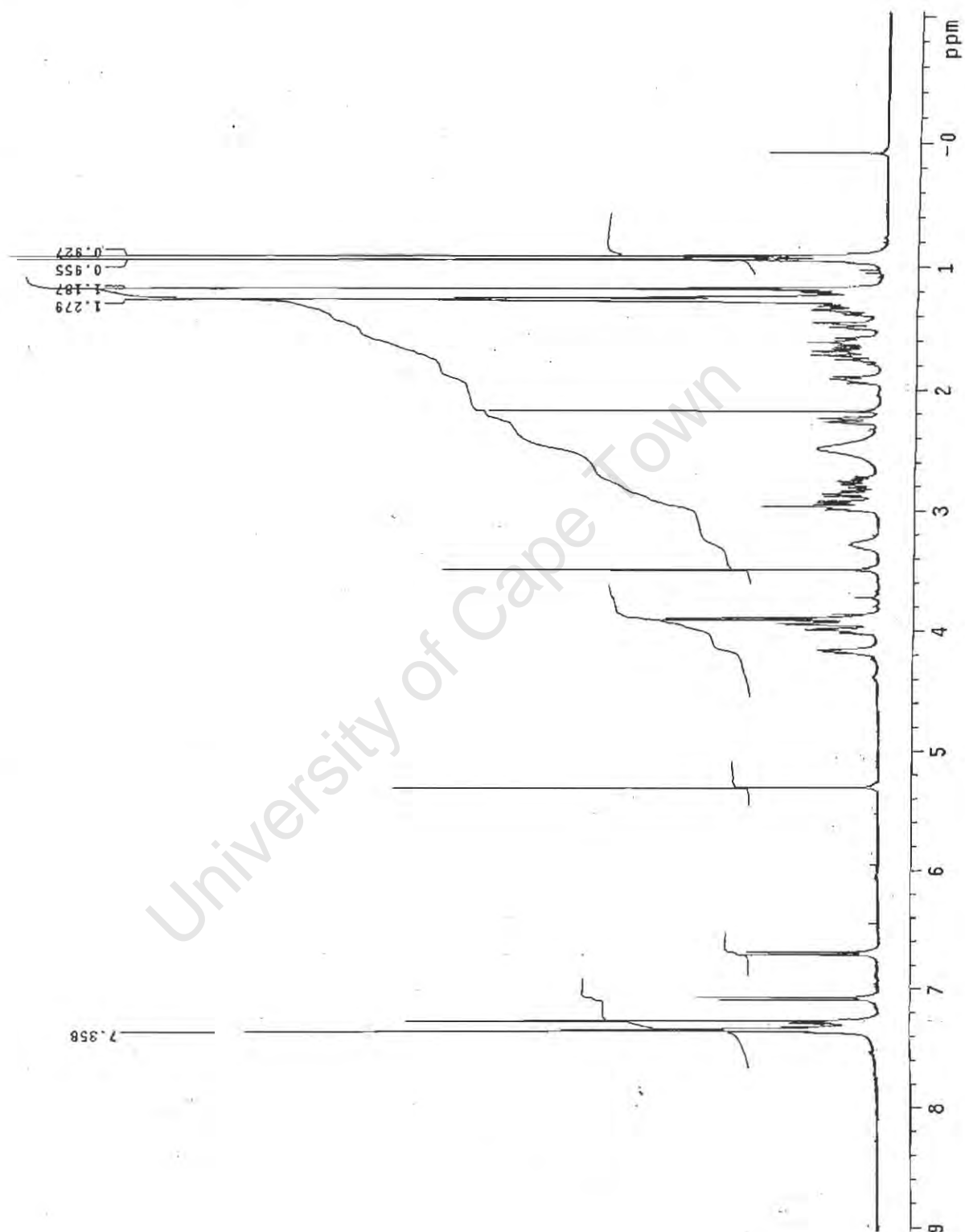
Figure A.3.5.1.: ^1H NMR of Compound 7

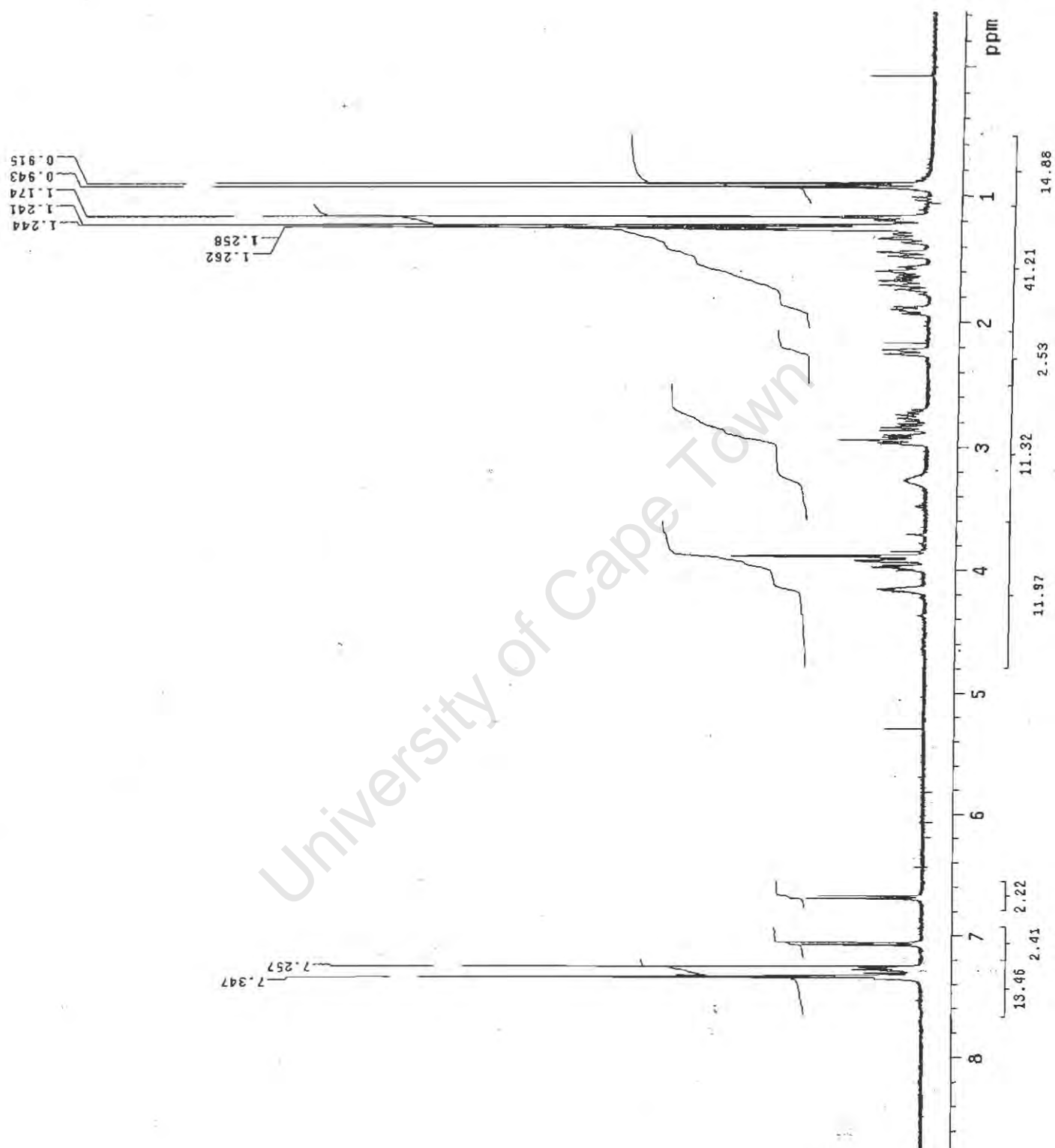
Figure A.3.5.2.: D₂O wash of Compound 7

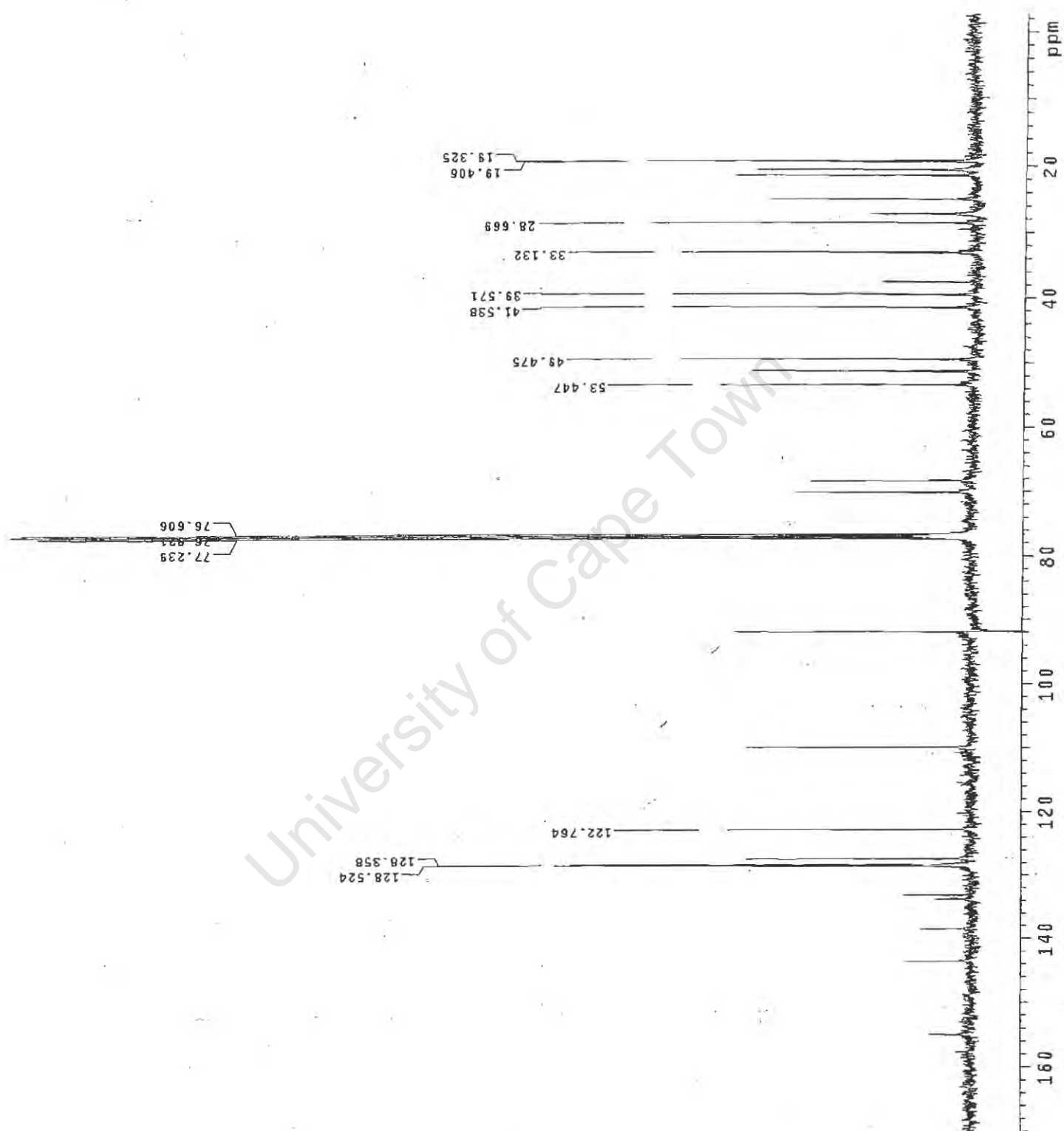
Figure A.3.5.3.: ^{13}C NMR of Compound 7

Figure A.3.5.4.: COSY of Compound 7

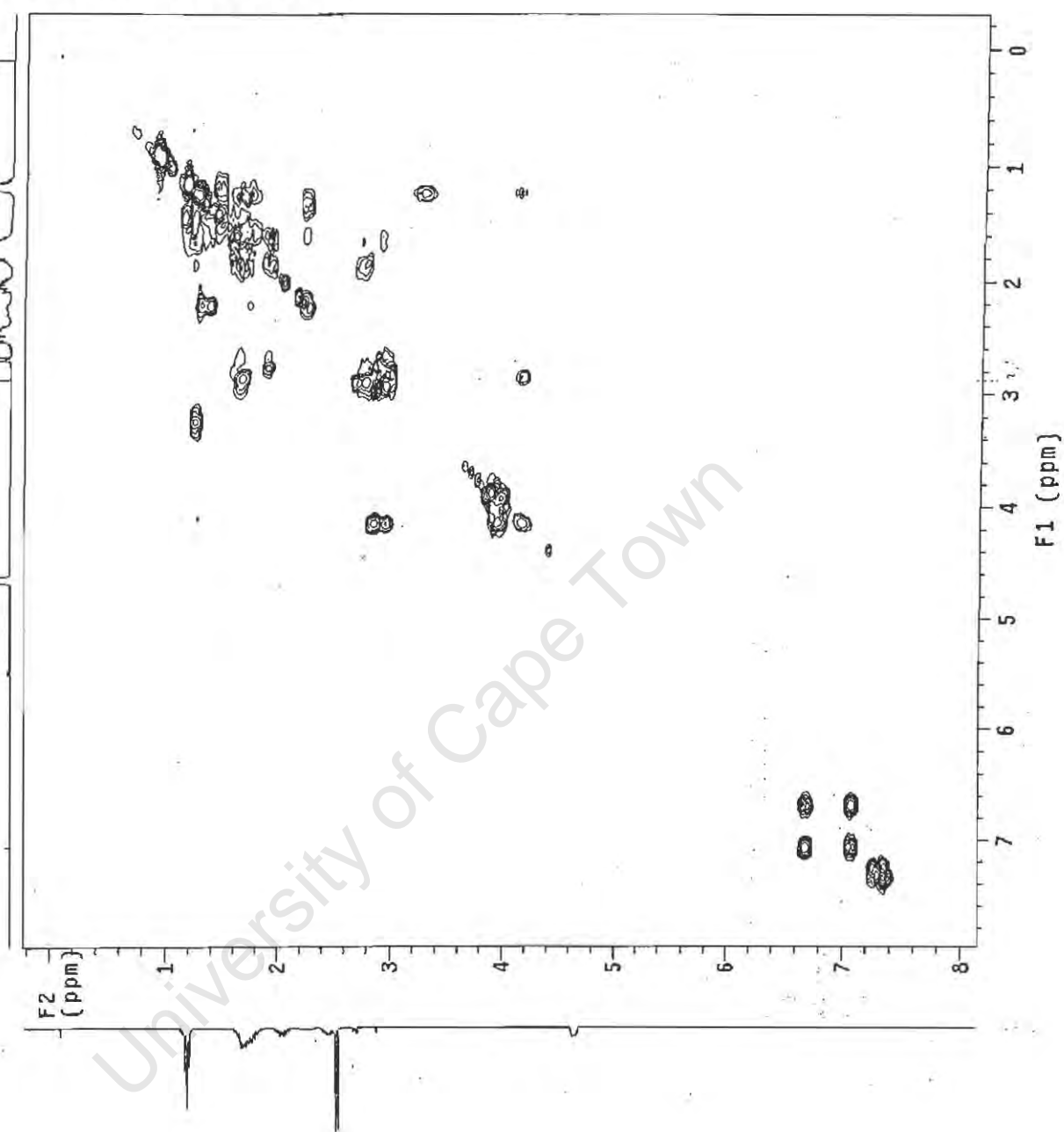


Figure A.3.5.5.: IR of Compound 7

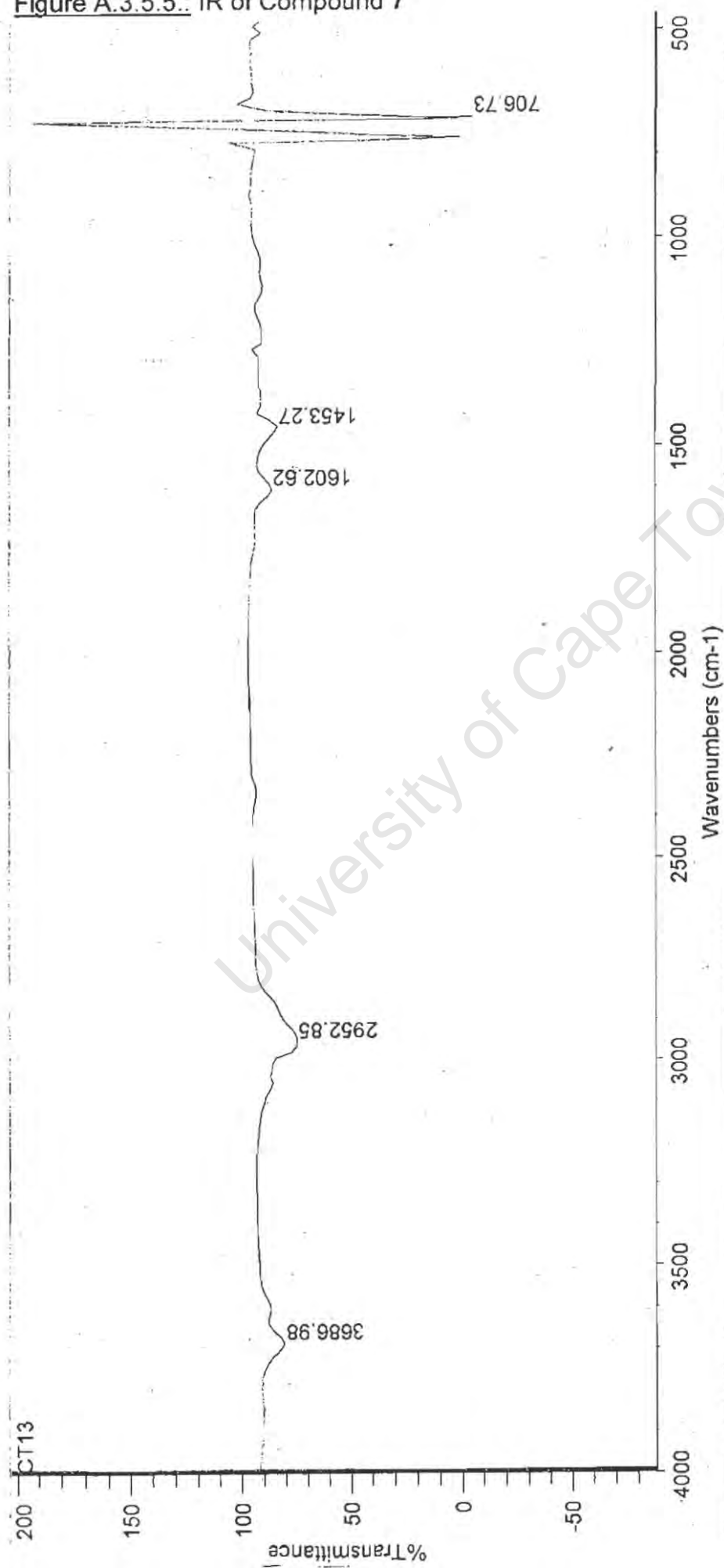
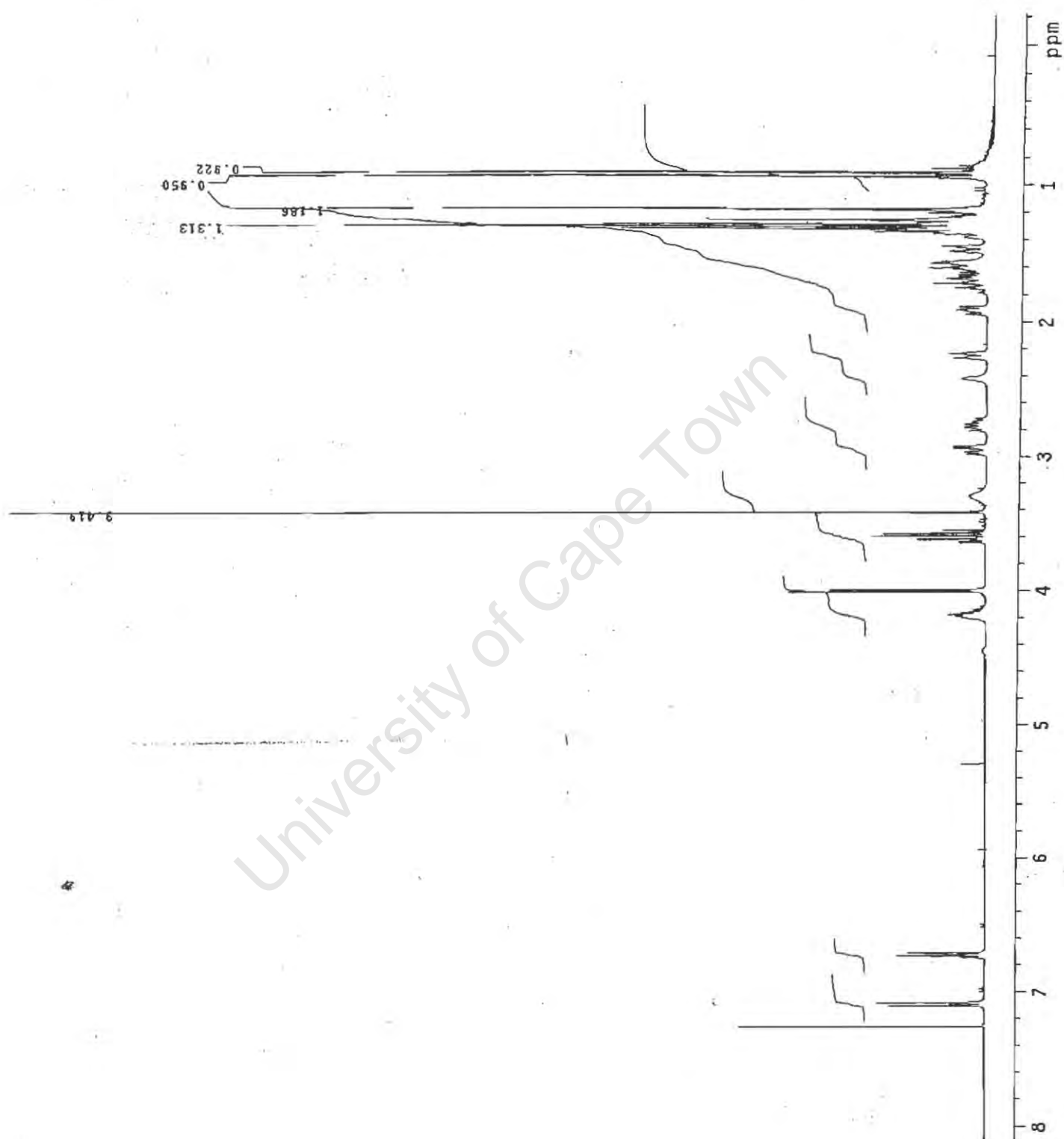


Figure A.3.6.1.: ^1H NMR of Compound 8

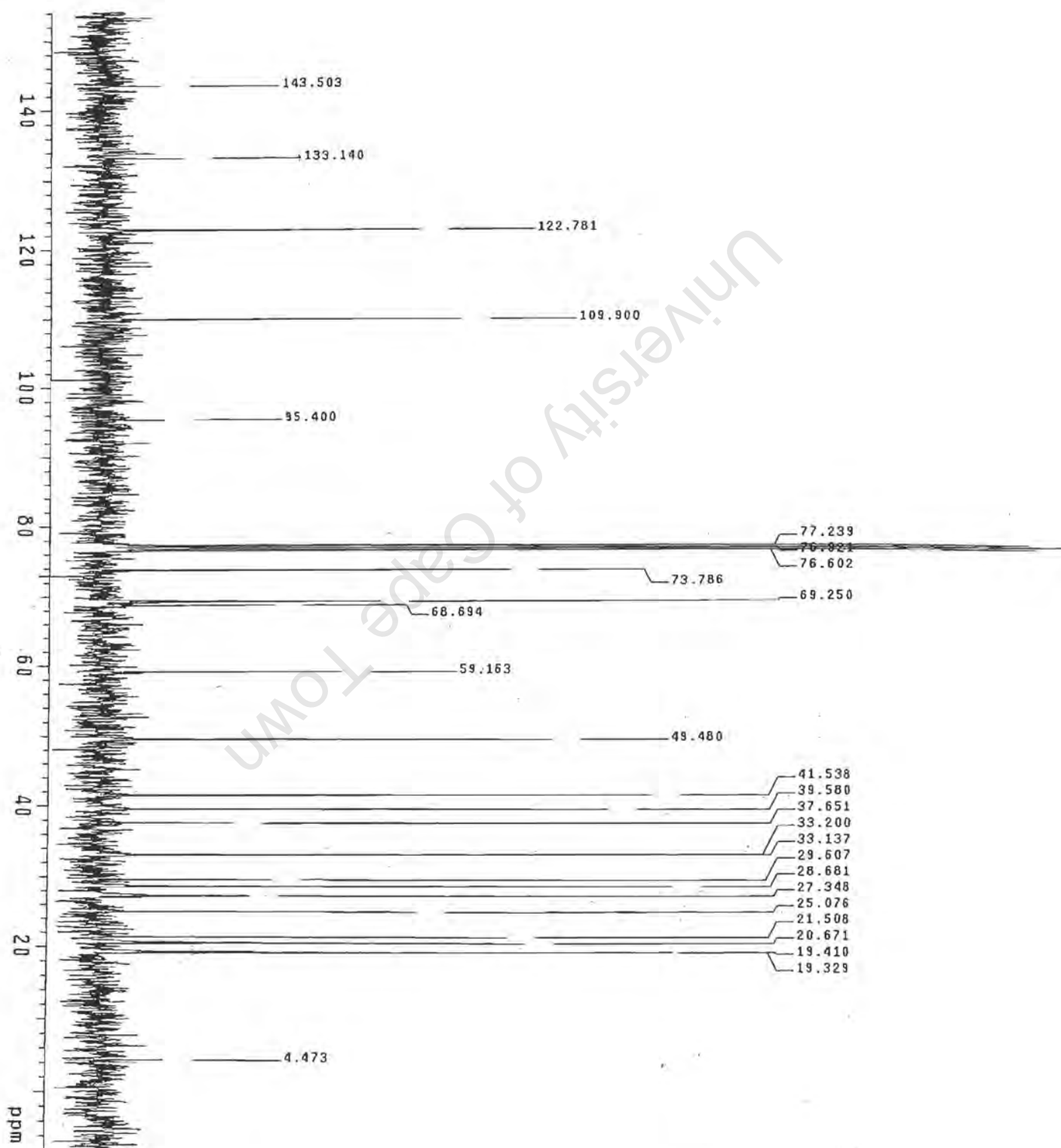
Figure A.3.6.2.: ¹³C NMR of Compound 8

Figure A.3.6.3.: IR of Compound 8

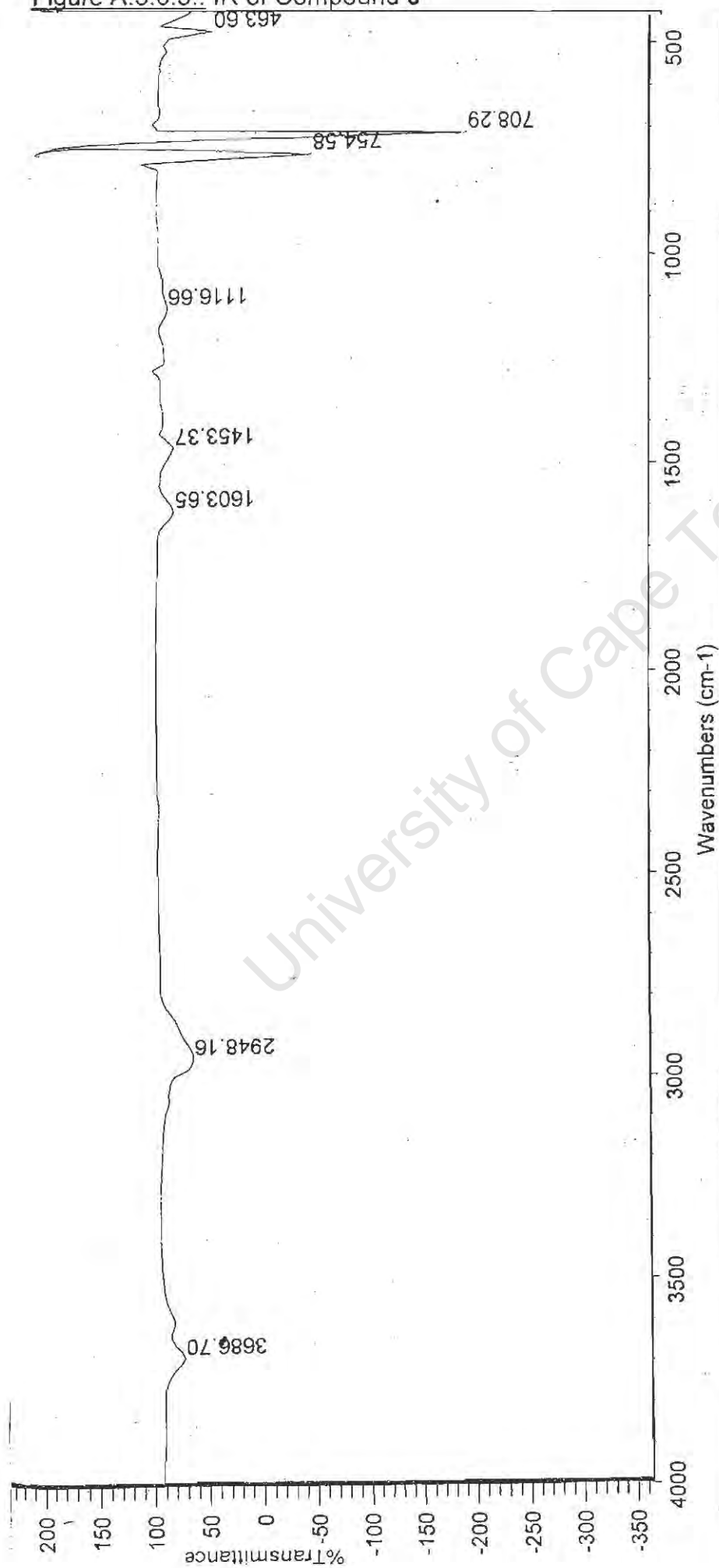


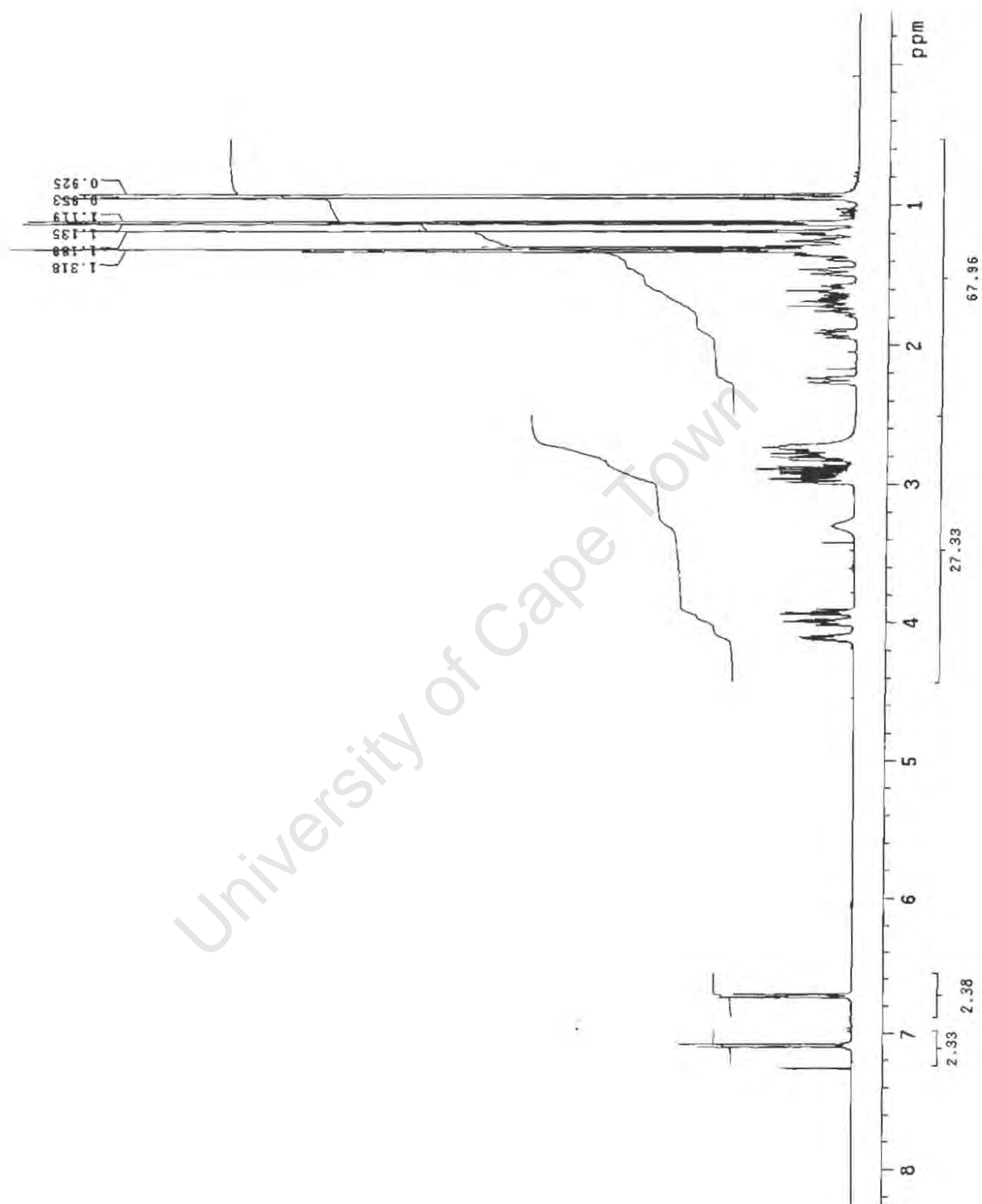
Figure A.3.7.1.: ^1H NMR of Compound 9

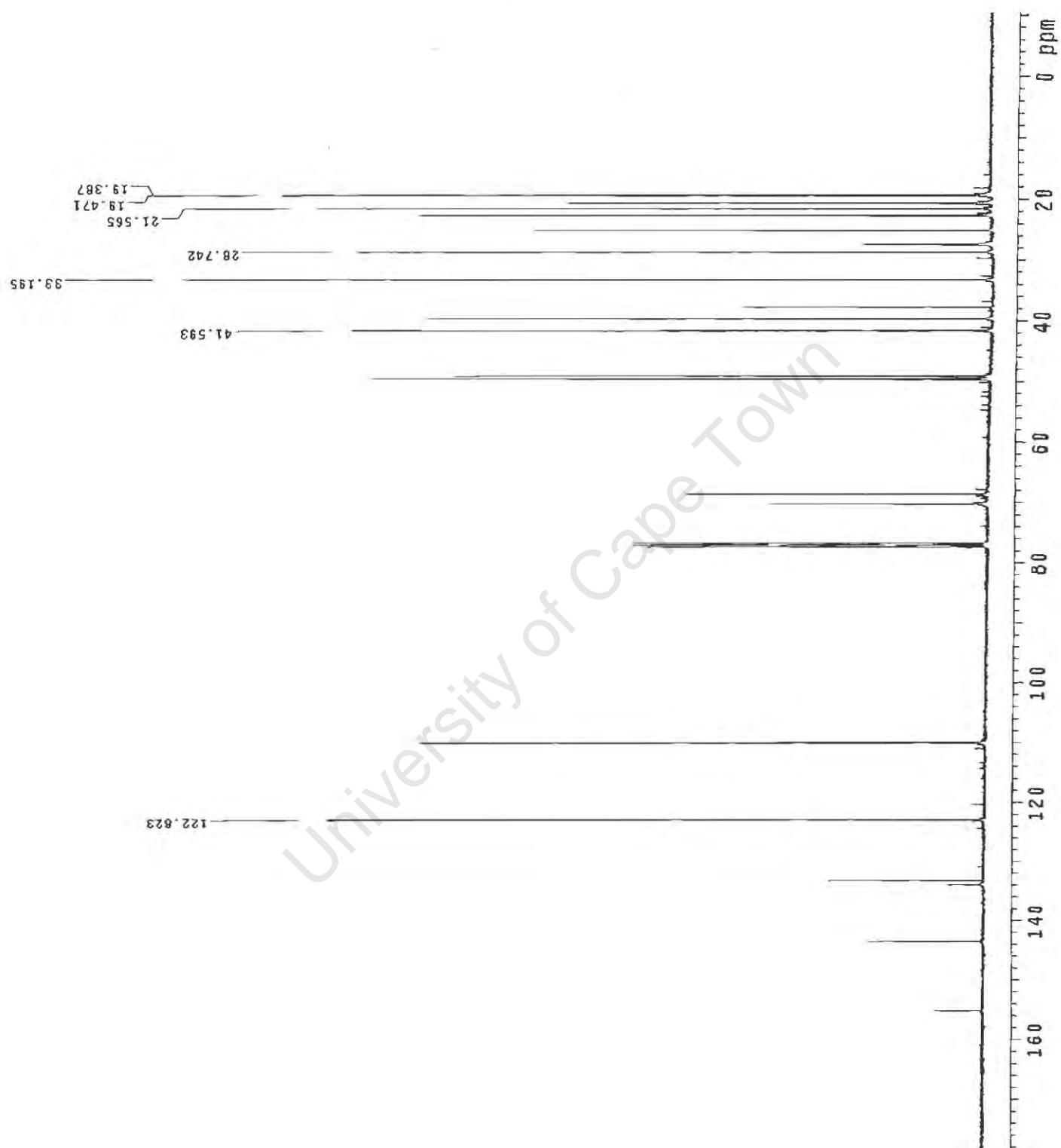
Figure A.3.7.3.: ^{13}C NMR of Compound 9

Figure A.3.7.3.: IR of Compound 9

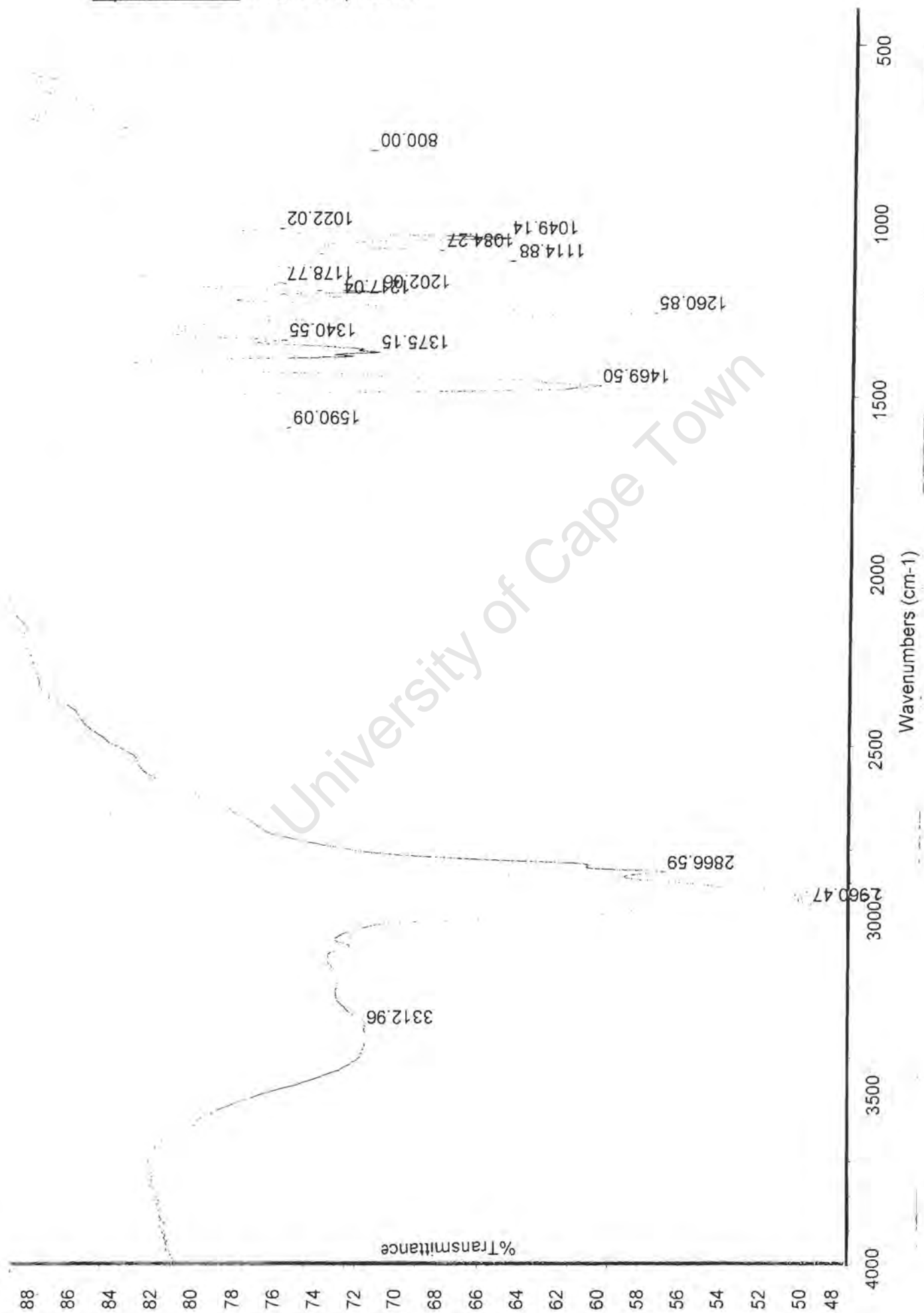


Figure A.3.8.1: ^1H NMR of Compound 16

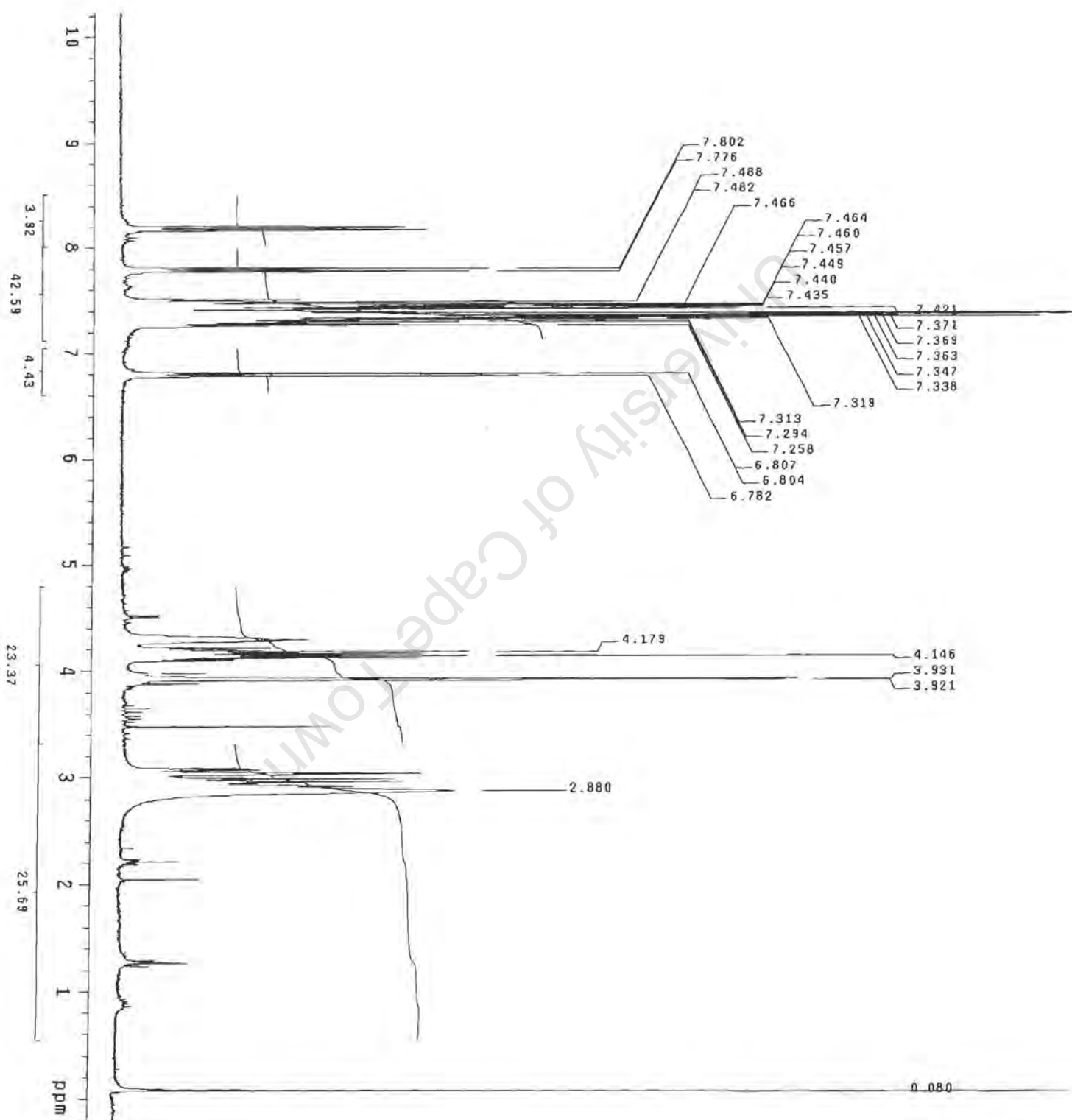


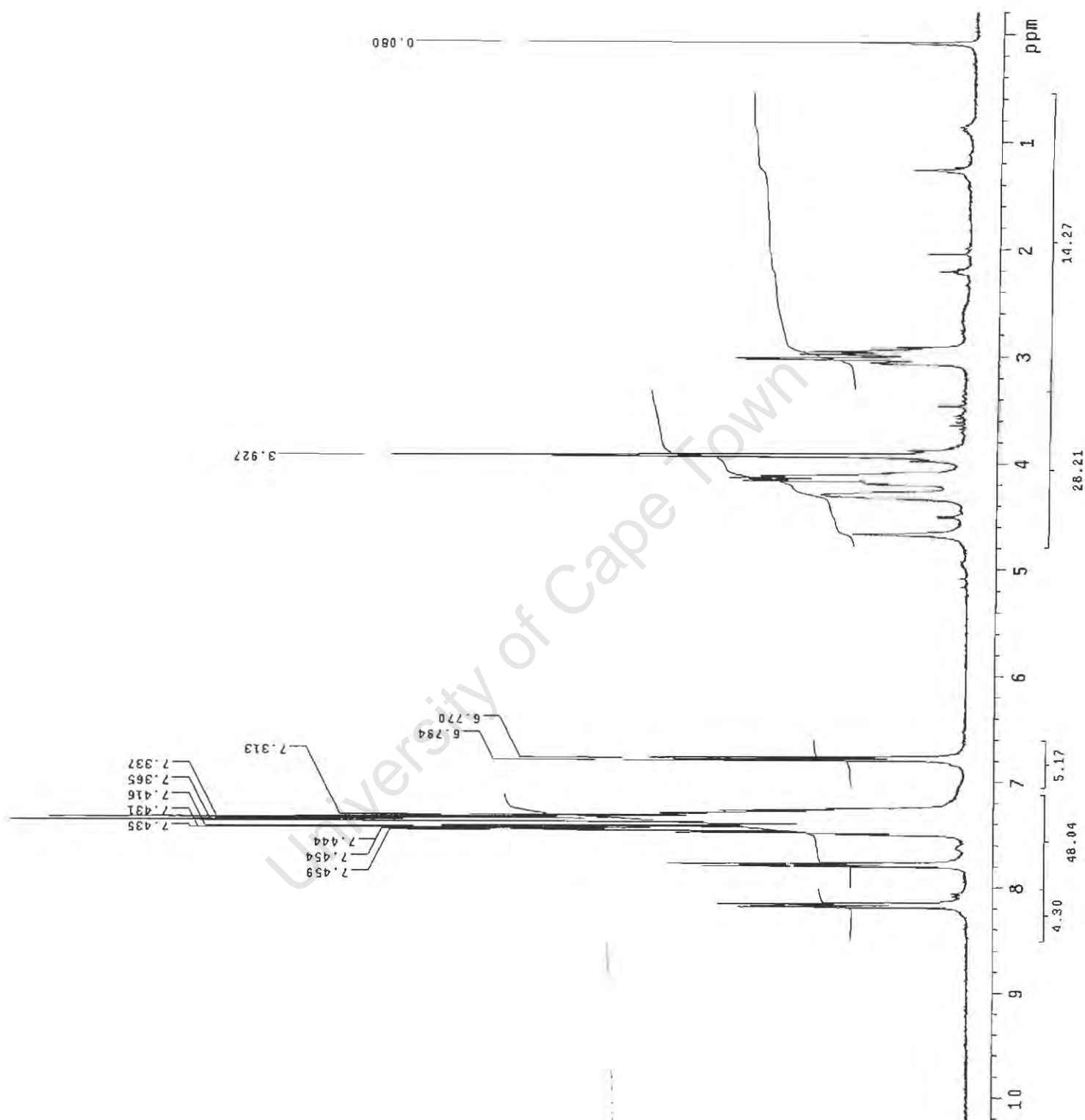
Figure A.3.8.2.: D₂O wash of Compound 16

Figure A.3.8.3.: ^{13}C NMR of Compound 16

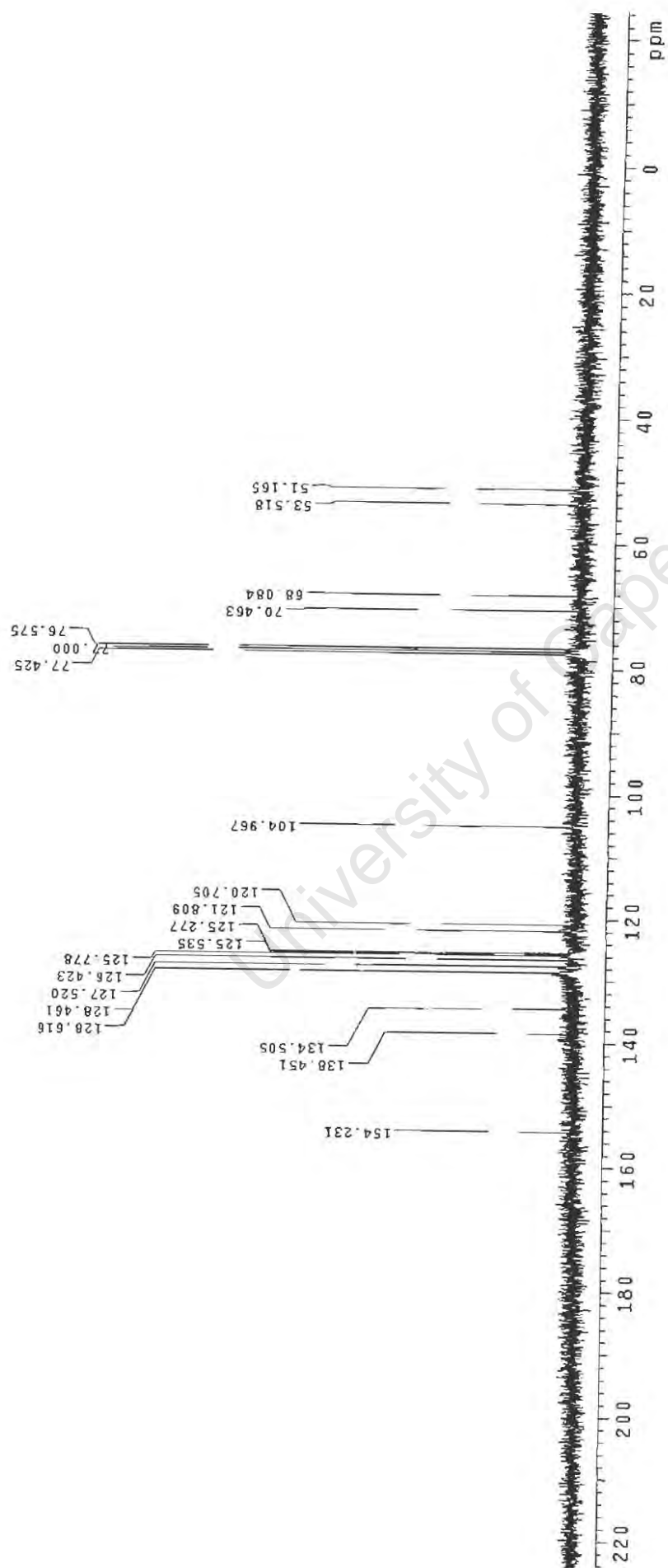


Figure A.3.8.4.: IR of Compound 16

